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PHYSIOLOGICAL ZOÖLOGY

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OXIDATION-REDUCTION INDICATOR PATTERNS IN THREE COELENTERATES

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THIS paper is a record of data concerning oxidation-reduction indicator patterns in the egg and early developmental stages of a hydrozoan and of an alcyonarian anthozoan and also of a scyphozoan scyphistoma and of the buds and stolons developing from it. It represents studies made at various times during past years which remained unpublished because it was hoped to continue work along this line either on the same or on other species. Since it now appears improbable that such an opportunity will arise and since the data concern large numbers of individuals and are definite in character as regards the patterns shown, it has seemed desirable to record them. They were obtained in part at the Scripps Institution of Oceanography, La Jolla; in part at the Hopkins Marine Station, Pacific Grove; and in part on material brought from the Hopkins Station to Palo Alto. The material and facilities provided by the marine laboratories are gratefully acknowledged.

MATERIAL AND METHODS

The calyptoblast medusa, *Polyorchis*, probably *P. montereyensis*, is abundant at times in the region of Pacific Grove and readily sheds eggs and spermatozoa; also the gonads with all stages of oöcyte development are available. The indicator studies on eggs and early development include the use of the indophenol or Nadi reaction and modifications of it, intracellular reduction of oxidized methylene blue and Janus green, and intracellular oxidation of these dyes in solutions reduced by sodium hydrosulphite. Janus green (diazine green) was used in various concentrations from 1/500,000 to 1/50,000 and methylene blue usually 1/50,000 but in some cases higher concentrations. The primary intracellular oxidation of Janus green from colorless to red of methylene blue in solutions reduced by hydrosulphite was used more extensively than staining by the oxidized dyes, followed by reduction by hydrosulphite because primary oxidation avoids the danger of differential injury by the oxidized dyes, with consequent alteration and even reversal of the real gradient pattern. Also there is less danger of injury in reduction following primary oxidation than in staining with oxidized dyes, because the intracellular oxidation of reduced dyes is much more rapid than staining by oxidized dyes and reduction is possible in a few minutes. However, intracellular oxidation and reduction of dyes in the *Polyorchis* material showed the same gradient pattern, except that

after periods of an hour or two in oxidized dyes more or less reversal of pattern occurred in some cases. The indophenol reaction was also used with concentrations of the reagents regarded for convenience in work of this kind as standard, and lower concentrations to one-quarter standard. With this reaction the same gradient pattern appeared as with primary oxidation of dyes. This reaction consists in the intracellular formation of the deep blue indophenol, catalyzed by an oxidase, usually believed to be cytochrome oxidase. Decrease of oxygen content of the solution is not necessary for the reaction, but intracellular indophenol is readily reduced to colorless by hydrosulphite with the same gradient pattern as in the reaction until differential injury occurs.[1]

Another source of interesting material for the study of indicator pattern consisted of developmental stages of the anthozoan, *Stylatula elongata*, one of the alcyonarian Pennatulacea, obtained from the soft mud of False Bay, San Diego. Since this is not a very well-known form, a brief description of the mature organism seems desirable. As collected, this form ranged in length from 10 to about 20 cm. The zoöids or polyps are small and are grouped transversely along the axis in successive so-called "leaves," so short that the organism can scarcely be regarded as branching. Proximal to the mature zoöids there is a band consisting of numerous transverse groups of zoöids in various stages of development. This band decreases in width proximally, almost to a point, and the stage of zoöid development is progressively earlier from its distal to its proximal end. At the extreme proximal end there is merely a thickening of tissue; somewhat farther distally the zoöid groups become distinguishable. Proximal to the band of developing zoöids is the smooth stalk, a region capable of contraction and of distention by water. The axial skeleton is a single, slender, apparently chitinous rod, extending 2–4 cm. beyond the most distal zoöids. There are also skeletal spines in connection with the zoöid groups. At low tide the only evidence of the presence of the animals is the bristle-like rod extending above the surface of the mud. The entire organism can easily be drawn out of the mud if the protruding skeletal bristle is firmly grasped, without preceding stimulation; but if the bristle is merely touched, the stalk usually contracts and even the bristle disappears. The animals can, of course, be dug out easily. *Stylatula* remains alive in the laboratory in clear running sea water and also lives for days in standing water. Large numbers of eggs were obtained from animals in the laboratory by natural shedding and were fertilized and developed. The eggs were extruded during the night and were found in cleavage or as blastulae in the morning. Eggs and growing oöcytes were also obtained by teasing groups of zoöids. Fertilizations were evidently successful during the day, but the eggs concerned were subjected to the indicators and not did continue development. Patterns of oöcytes at various stages of growth, of full-grown, and of developmental stages up to the primary polyp and the first bud arising from it were investigated by intracellular reduction and reoxidation of methylene blue in various concentrations from 1/100,000 to 1/20,000 with a wide range of exposure periods and large amounts of material.

In the course of work with this organism the question arose concerning the relations between the mature zoöids and the band of developing zoöids. Do the mature zoöids degenerate at the distal

[1] The preparation and use of the indophenol reagents in work of this sort have been described in various papers, e.g., Child, 1947, 1949; and others.

end of the axis with replacement by zoöid groups from those in process of development? For various reasons it seemed highly probable that this must be the case. Any other significance for the presence of these zoöids in all stages of development could scarcely be assumed. In many collections of material it was noted that the total length of the organism was in no case much greater than 20 cm. However, if all the groups of mature zoöids remained alive and all the many groups of developing zoöids attained complete development, even if no more zoöid groups appeared than those visible at any one time, the total length of the organism must become much greater than 20 cm. Moreover, in all cases in which eggs were naturally extruded in the laboratory, they came from zoöids near the middle of the fully developed groups, not from the distal end. This was evident because the animals do not move about in the containers and the shed eggs stick to the bottom. Similarly, when eggs or oöcytes were obtained by teasing zoöid groups, they were always found in groups from the middle region or proximal to it. In short, the sexually mature zoöids were not at the distal end of the axis but near the middle. Also, zoöids at the distal end of the axis often appeared to be degenerating or undergoing involution. Search of the literature available gave no information on this question beyond the statement that the primary polyp often degenerated. However, in reply to a letter of inquiry, the following personal communication was received from Dr. Elizabeth Deichmann, of the Museum of Comparative Zoölogy, Cambridge, Massachusetts, an authority on Alcyonaria: "As far as I know, nothing is known about the growth of these forms, but it seems certain that polyps constantly are worn off from the top and replaced from the growth-area near the base." At present there seems to be no evidence for any other conclusion.

In the third form included in this paper, the scyphistoma of *Aurelia lobata*, embryonic material was not available. The patterns of intracellular reduction and reoxidation were determined in the fully developed nonstrobilating scyphistoma and in the buds and stolons. Large amounts of material were available, and the animals remained in good condition, producing stolons and buds almost indefinitely in running water in the laboratory. The material was first obtained during a period at the Scripps Institution of Oceanography, La Jolla, California, on shells from the bottoms of ships undergoing cleaning. A year later it was found near Pacific Grove in great abundance underneath a boathouse float and on the lower sides of rocks in the water but not in contact with the bottom. This form is not highly susceptible to oxidized methylene blue, except in strong light, or to oxidized Janus green. Only patterns of intracellular reduction and reoxidation of these two dyes were determined in this material. Methylene blue 1/20,000 and 1/10,000 and Janus green 1/100,000 and 1/50,000 with wide ranges of staining periods were satisfactory. The animals remained alive for days in various concentrations of methylene blue, except for some disintegration of tentacles. Later attempts to obtain this material in the region of Pacific Grove were unsuccessful.

At the time when the patterns of *Stylatula* and of the *Aurelia* scyphistoma were investigated, sodium hydrosulphite had not, so far as the writer is aware, been used as an agent for reducing oxidation-reduction indicators, either in solution or in the cells of organisms. The agents commonly used for reduction, e.g., hydrochloric acid with sodium thio-

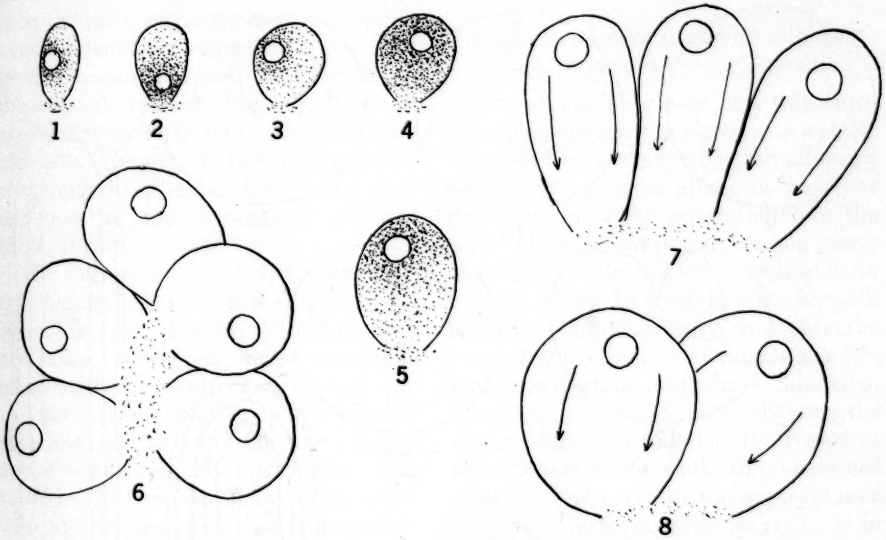
sulphite, and "rongalite," a preparation containing formalin and thiosulphite, are so extremely toxic that there is always great danger of differential injury with differential retardation of intracellular reduction or even reversal of the real pattern. Some of the earlier papers on differential intracellular reduction of indicators provide examples of errors resulting from the use of these toxic reducing agents (Child, 1941, note on p. 92). The writer used these reducing agents in some earlier work, but they were regarded as highly unsatisfactory because of their toxicity. In more recent studies of indicator patterns, sodium hydrosulphite has proved to be an extremely useful reducing agent, first, because it is not appreciably toxic, even in concentrations far above those necessary for intracellular reduction; second, because reduction in the external solution occurs almost at once; and, third, because intracellular oxidation in the reduced solution and intracellular reduction can usually be repeated, often several times, before intracellular dye concentration becomes so high that the gradient pattern is altered by differential injury. For the rather highly susceptible *Stylatula*, only methylene blue was used because of its relatively low toxicity. For the much less susceptible scyphistoma Janus green was also used. The data on pattern in *Polyorchis*, obtained very recently and with the use of hydrosulphite as reducing agent and of the indophenol reaction, as well as intracellular oxidation and reduction of dyes, have a broader experimental basis than the earlier data. However, intracellular reduction of the dyes by oxygen uptake of the material sealed in a small amount of dye solution in the earlier work was effected without the use of a toxic reducing agent and could usually be repeated after reoxidation without alteration of the pattern until intracellular dye concentration became very high. With this method intracellular dye reduction is, of course, less rapid than with hydrosulphite, and provision for uniform distribution of oxygen, by frequent change of position of the material in the sealed preparation, must be made. The large amounts of material used for oxidation-reduction in both *Stylatula* and the scyphistoma, with wide ranges of staining periods, and the uniformity of the patterns observed until obvious injury with alteration or reversal of pattern or absence of reduction occurred are believed to exclude possibility of error. It should be noted that for determination of the reduction patterns of the entire mature *Stylatula* the whole organism, after staining by oxidized methylene blue, was sealed in oxidized dye solution in a glass tube a few centimeters longer than the animal and just large enough to permit movement back and forth through the solution in order to avoid local differences in oxygen content of the solution. Here, also, intracellular dye reduction resulted from oxygen uptake of the animal.

The figures are diagrammatic outlines without details of structure. The direction of progress of intracellular reduction or oxidation is usually indicated by arrows pointing from the region of more rapid, to that of less rapid, change. Longer arrows indicate a greater differential, i.e., a steeper gradient; shorter arrows, less differential. Stages of differential reduction in some oöcytes and egg are indicated by graded shading. Except for oöcytes and eggs, the figures of the same organism are approximately on the same scale.

GRADIENT PATTERN IN THE *Polyorchis* EGG AND IN ITS EARLY DEVELOPMENT

Oöcytes in all stages of growth were readily obtained from the gonads of females. In the earlier oöcytes the posi-

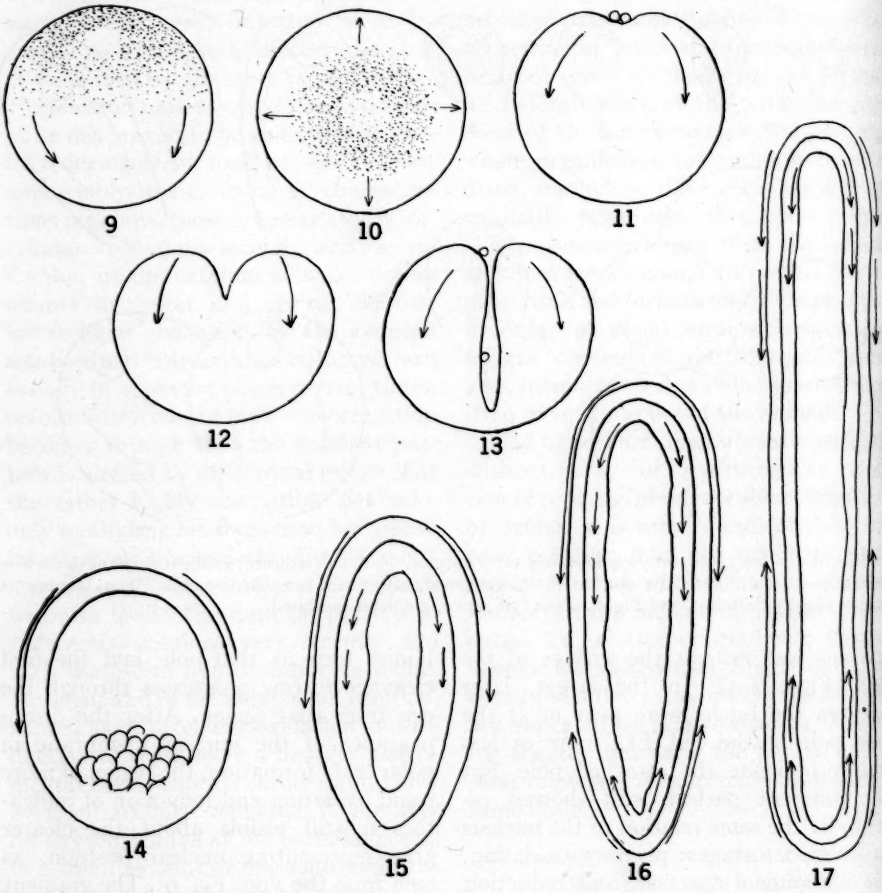
tion of the nucleus was highly variable; in some cells it was near the attached end (Fig. 1) and often near one side at different levels (Figs. 2 and 3) or near the unattached pole (Figs. 4 and 5). Primary intracellular oxidation of Janus green from a solution reduced by hydrosulphite was more rapid than elsewhere in a zone about the nucleus and decreased in rate with increasing distance from the nucleus and was most rapid where the nuclear position in advanced oöcyte stages at the tip of a gonad; Figure 7 nuclear positions and gradient direction in cells slightly flattened under a cover glass; and Figure 8 nuclear positions and gradients in two approximately full-grown oöcytes, the general gradient pattern being indicated by arrows in Figures 7 and 8. In the full-grown egg, as discharged from the gonad, the nucleus is still near one pole, the polar



FIGS. 1–8.—*Polyorchis* oöcytes. Figs. 1–5, early oöcytes, relation of indicator gradient patterns to varying nuclear positions. Fig. 6, later oöcytes at tip of a gonad, with nuclei near the free pole. Fig. 7, late oöcyte stages, slightly flattened, and Fig. 8, about full-grown, gradients indicated.

nucleus was nearest the surface of the cell (Figs. 2–5). In the larger, later oöcytes the nuclei were near or at the free pole of the cell, i.e., more or less nearly opposite the attached pole, but the gradient pattern still showed essentially the same relation to the nucleus as in earlier stages: primary oxidation, the indophenol reaction, and reduction were most rapid where the nucleus was nearest the surface of the cell and in the cell generally, decreased from a zone about the nucleus. Figure 6 shows bodies form at that pole, and the first cleavage furrow progresses through the egg from that region. After the disappearance of the nuclear membrane in polar body formation, the region of more rapid oxidation and reduction of indicators is still visible about the clearer area representing nuclear position, as seen from the side (Fig. 9). The gradient pattern is so distinct that it can also be clearly seen in surface view from the apical pole (Fig. 10). In Figures 11, 12, and 13, showing polar bodies and stages

of the first cleavage, the earlier perinuclear zone of more rapid oxidation and reduction does not move to the center of the cell with the nucleus but retains its position and becomes the primary gradient of later stages. In Figure 14, a stage of immigration of cells forming the entoderm, and in Figure 15, a slightly later planula stage, this gradient is distinct. The planula entoderm, originating from the basal polar region (Fig. 14), has become an entodermal layer in Figure 15. It oxidizes and reduces less rapidly than ectoderm, as might be expected, unless it has undergone marked change in condition after immigration because of its origin from the region of least rapid oxidation and reduction of earlier stages. However, a slight oxidation-reduction gradient appears to be present in it with basipetal decrease, i.e., in the same direction as the ectodermal gradi-



FIGS. 9–17.—*Polyorchis*, later developmental stages. Figs. 9 and 10, gradient pattern after disappearance of nuclear membrane. Figs. 11–13, first cleavage, gradients indicated. Figs. 14 and 15, early planulae, with primary gradient pattern. Figs. 16 and 17, development of secondary gradient in later planulae. The original "anterior" end of the swimming stage is uppermost in all figures.

ent, as indicated by the shorter arrows in the entoderm of Figure 15. How this entodermal gradient originates remains uncertain. Possibly the cells which migrate farthest from the basal region become more active than those which migrate shorter distances, or perhaps the greater activity of the more apical ectodermal levels induces the entodermal gradient pattern.

As the planula elongates, the gradient pattern becomes less distinct, particularly in the basal or posterior region; and a new gradient soon makes its appearance in this region, opposed in direction to the original gradient (Fig. 16). This new gradient is at first short but gradually increases in length. A slight new gradient also appears in the entoderm in the same direction as the ectodermal gradient (Figs. 16 and 17).

The *Polyorchis* planula becomes greatly elongated, slender, and almost wormlike (Fig. 17). Its swimming activity decreases; many of these elongated planulae become attached by one end and more or less erect. In many cases the attached end is certainly the original apical or anterior end, but there is often so little difference in diameter or otherwise at the two ends that it cannot be determined with certainty which end became attached, though it is regarded as probably the original apical end in all cases. The question of the significance of the second gradient in the planula and of the pole of attachment is taken up in the discussion.

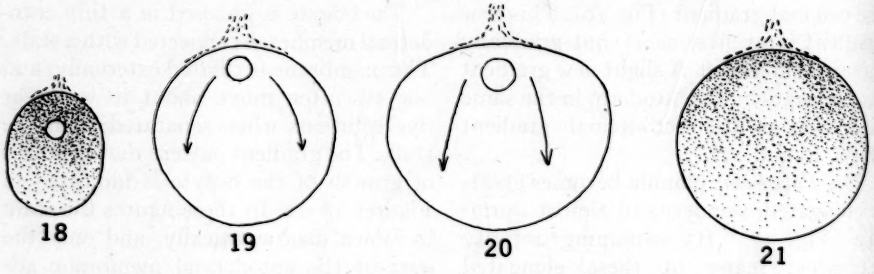
Methylene blue 1/50,000 and higher concentrations were somewhat toxic with exposures of ½–1 hour or more. On reduction by hydrosulphite, the gradient pattern was often reversed. In oöcytes reduction progressed toward, rather than away from, the nuclear region, and some cells did not reduce at all. Similar reversals of the reduction pattern or failure to reduce occurred with high dye concentrations in later stages; but with lower concentrations of oxidized methylene blue and with Janus green 1/200,000 and 1/100,000 uniform results as regards reduction were obtained. With standard concentrations of the reagents the indophenol reaction also gave the same uniform results as did primary dye oxidation from reduced solutions, and repeated reduction and reoxidation of intracellular indophenol was usually possible.

GRADIENT PATTERNS IN THE EGG AND EARLY DEVELOPMENT OF *Stylatula*

The oöcyte is inclosed in a thin entodermal membrane connected with a stalk. The membrane is ciliated externally, and oöcytes often move about in water or dye solutions when separated from the stalk. The gradient pattern during stages of growth of the oöcyte is indicated in Figures 18–20. In these figures the stalk is shown diagramatically, and only the part of the entodermal membrane adjoining the stalk is similarly indicated. Except in some very early oöcytes, the nucleus has been found in the region of the cell nearest the stalk. In no case has a nucleus been seen at the opposite pole of the cell farthest from the stalk, as in oöcytes of many other organisms. The growing oöcyte becomes brownish in color and almost opaque. The rate of intracellular dye reduction decreases from a zone about the nucleus (Figs. 18–20). In some full-grown oöcytes the nuclear membrane disappeared, leaving a relatively clear space, but the reduction pattern remained the same. In Figure 21 this pattern is indicated by graded shading instead of by arrows, the depth of shading decreasing with rate of reduction. These eggs were doubtless approaching polar body formation, but after staining and decrease of oxygen for the following reduction, actual forma-

tion of polar bodies did not occur. In another lot of eggs removed from one animal the nuclear region was in the center of the egg, but the nuclear membrane had disappeared before dye reduction. These were evidently approaching the first cleavage, but cleavage did not occur after staining and reduction. In these eggs as in those of *Polyorchis*, the gradient pattern did not change in position as the nucleus moved to the center of the cell but remained as the primary polar gradient. Intracellular reduction occurs rapidly in the eggs when several pear, or some eggs failed to reduce. Also after $\frac{1}{2}$–1 hour in methylene blue 1/20,000 or 1/10,000 there may be differential injury and reversal of the gradient on the first reduction. In all cases the oöcyte reduced much more rapidly than did the tissue of the stalk.

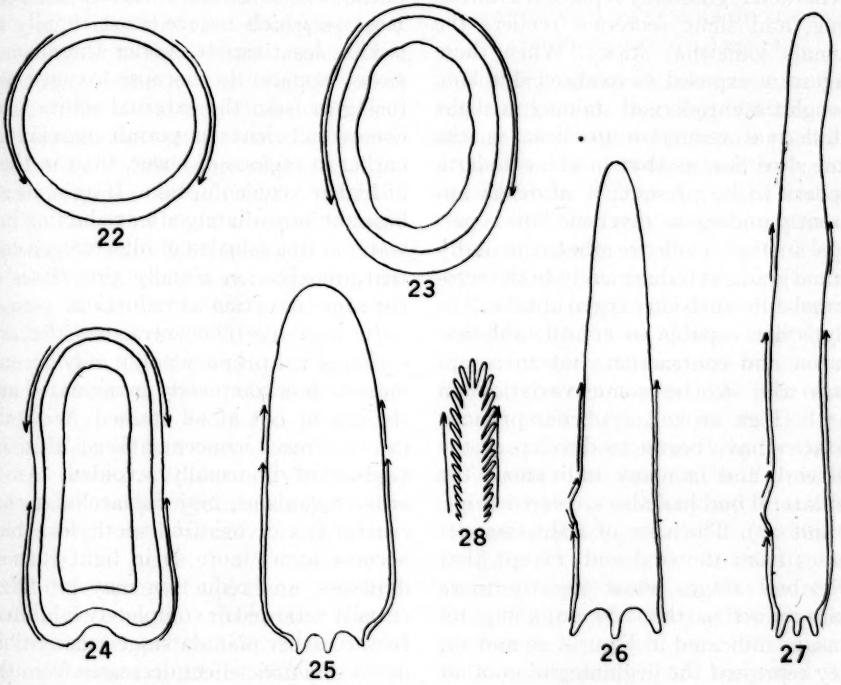
The same gradient persists during cleavage and in the solid blastula; but, with formation of entoderm by delamination and further division, the gradient appears chiefly in the ectoderm, at least in part because the entoderm stains much less rapidly, perhaps also because the ectoderm arises from those parts of the cells nearer the external surface and including the external cortical region. In a spherical preplanula stage with slightly thicker ectoderm at the pole which is posterior in swimming, a very distinct reduction gradient is present in the ectoderm, decreasing in rate from the thinner anterior to the thicker posterior pole (Fig. 22). This is the same gradient as in earlier stages. The spherical preplanula gradually elongates to form the early planula without essential change in the gradient pattern (Fig. 23). Even in the higher dye concentrations, entoderm stained very slowly, and the reduction gradient was slight and in the same direction as the gradient in the ectoderm



FIGS. 18–21.—*Stylatula*. Figs. 18–20, oöcytes with nucleus near the stalk and rate of reduction decreasing from a zone about it. Fig. 21, reduction pattern after disappearance of nuclear membrane in the egg.

are sealed in a small volume of solution, and on re-exposure to air there is rapid reoxidation, usually with a gradient opposite in direction to the reduction gradient, as in many other organisms when reduction is brought about by oxygen uptake of the animals or when an excess of hydrosulphite is used for reduction. Under these conditions oxygen rather than oxidase activity becomes the factor determining the rate of reoxidation. Oxygen diffusing into the cell becomes sufficient to permit reoxidation earlier in regions of less, than in those of more rapid, oxygen uptake. Reduction was repeated three times on some lots of eggs. With the third reduction, reversals of gradient pattern, evidently resulting from differential injury, began to ap-

preceding differential injury. During the second day of development a second gradient began to appear in the planula, with the region of most rapid reduction at the posterior pole and opposed in direction of decrease in rate of reduction to the primary gradient (Fig. 24). This new gradient increased in length and progressively replaced the primary gradient over most and perhaps over the entire length of the planula. Meanwhile, the primary gradient became less distinct, and the differential in the new gradient became so slight at its lower levels that it was often difficult to determine exactly how far it did extend. During these stages an invagination appeared and became deeper at the high end of the new gradient, forming the mouth and stomodeum (Fig. 24). The posterior end of the swimming planula, the region of least rapid reduction in earlier stages, has now become the region of most rapid reduction and the oral end, while the region of most rapid reduction in the original gradient is now the region of least rapid reduction and the aboral end. In Figures 25, 26, and 27 the new gradient pattern is indicated



FIGS. 22–28.—*Stylatula*, later developmental stages. Fig. 22, preplanula, with primary reduction gradient decreasing in rate from thinner to thicker ectodermal pole. Fig. 23, early planula, with primary reduction gradient decreasing in rate from original "anterior" pole of swimming stages. Fig. 24, later planula, with primary and secondary gradients and invagination of mouth and stomodeum at "posterior" end. Fig. 25, early primary polyp, with secondary gradient extending over most of the body-length and tentacles developing about the oral region at its high end. Figs. 26 and 27, still later stages of primary polyps, secondary gradient extending over most of the body, early stage of first lateral bud showing beginning of a new gradient pattern. Fig. 28, pinnate tentacle of mature zoöid, indicating acropetal decrease in general reduction gradient pattern in association with order of development of pinnules, a reversal of the basipetal decrease in rate of reduction in early tentacle buds, as indicated in Figs. 25–27.

over the oral half or more of the organism, which is now becoming the primary polyp; but toward the aboral end no gradient is shown because it remained uncertain whether the new gradient extended to the extreme aboral end or whether a trace of the primary gradient persisted there. With reversal of the gradient pattern, free-swimming activity decreases, but, when swimming occurs, the aboral end is still "anterior." Evidently the direction of effective beat of the cilia has not undergone reversal. Contractions, often more or less peristaltic in character, gradually replace free swimming, and slime secretion renders the animals somewhat sticky. When these stages are exposed to oxidized dye long enough for entodermal staining, a slight entodermal reduction gradient in the same direction as that in the ectoderm appears to be present; it also has apparently undergone reversal. This is perhaps further evidence that the entodermal gradient is due merely to the ectodermal differential in oxygen uptake. The polyps are capable of considerable extension and contraction, but there appears also to be some variation in length (Figs. 26 and 27). Four primary tentacles have begun to develop at the oral end, and in many individuals the first lateral bud had also appeared (Figs. 26 and 27). The rate of reduction decreases from the oral end, except that early bud stages show slightly more rapid reduction than do adjoining regions, as indicated in Figures 26 and 27. They represent the beginning of another new gradient pattern. Development of further buds was not observed under laboratory conditions. Burrowing into the mud would probably begin under natural conditions at about the stages of Figures 26 and 27.

The planula stages and the primary polyp apparently have a rather high oxygen uptake. Sealed in small volumes of dye solution, they usually begin to reduce in a few minutes, and often, when a few stick together, reduction occurs in open solution. Motor activity soon ceases when they are sealed; but, after intracellular reduction and loss of motility, complete recovery is often possible on exposure of the solution to air. With slight or gradual aeration after reduction, the reoxidation gradient is more rapid in regions of less, than in those of more rapid, reduction, i.e., as noted above for earlier stages, the reoxidation gradient is the reverse of the reduction gradient. Regions which reduce most rapidly reoxidize least rapidly under these conditions, apparently because oxygen diffusing in from the external solution becomes sufficient to permit reoxidation earlier in regions of lower, than in those of higher, oxygen uptake. If animals are brought immediately after reduction into water or dye solution of high oxygen content, reoxidation usually progresses in the same direction as reduction.

In high dye concentrations the oral region of the primary polyp may become more or less completely invaginated and slightly or not at all stained. With the use of lower concentrations, this invagination is usually avoided. As in other organisms, high intracellular concentrations of oxidized methylene blue become toxic, more so in light than in darkness, and reduction may be differentially retarded or completely inhibited. In the earlier planula stages susceptibility to this toxic effect decreases from the high end of the primary gradient. In stages when two gradients are present (Fig. 24) reduction may be most retarded at both ends, and in the later planula and the primary polyp it is most retarded at the oral end.

Certain characteristics of reduction pattern in the mature organism are also

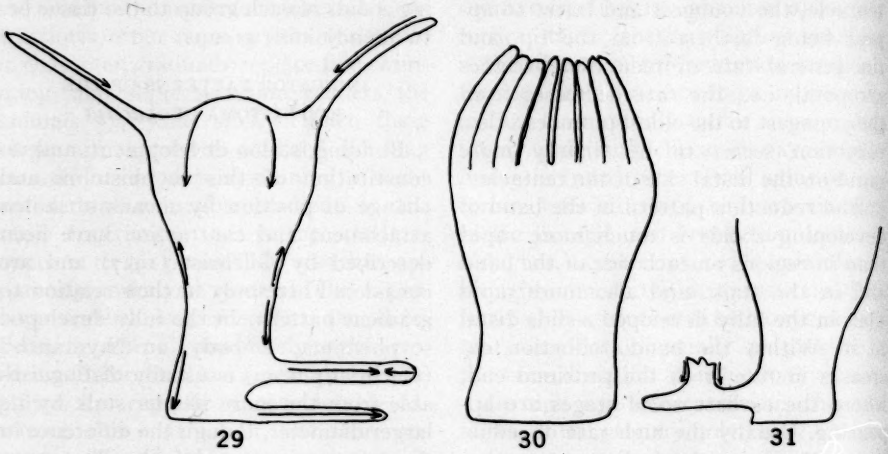
of interest. Reduction in the naked portions of the single fully developed tentaculate zoöids (autozoöids) decreases in rate basipetally from the mouth region. In the early tentacle buds of the primary polyp, the rate of reduction decreases basipetally (Figs. 26 and 27), as in other coelenterates, but in later stages the tentacles become pinnately branched by development of "pinnules" laterally on each side. The development of pinnules progresses basipetally from the tip of the tentacle, the youngest and latest to appear being farthest from the tip, and the general rate of reduction becomes acropetal, i.e., the rate decreases from the youngest to the oldest pinnules. Also, reduction seems to be slightly more rapid on the distal side of the tentacle.

The reduction pattern in the band of developing zoöids is much more rapid than in regions on each side of the band and in the stalk, and also more rapid than in the fully developed zoöids distal to it. Within the band, reduction decreases in rate from the proximal end, where the earliest zoöid stages are appearing. Usually the high rate of reduction extends a short distance farther proximally, where zoöid groups are not distinguishable but where there is doubtless increase in cellular activity preceding visible morphogenesis of zoöids. The decrease in rate distally in the band parallels, in general, the progress of zoöid development, and there is a slight further decrease in rate in the "leaves" of fully developed zoöids, though with little difference in rate at different levels of this region. Also in the band of developing zoöids each transverse zoöid group, i.e., the developing leaf with its zoöids, is separated from other groups distal and proximal to it by a short, but distinct, space. In this space siphonozoöids develop sooner or later, but evidently their development does not determine any such increase in rate of reduction as does development of the tentaculate zoöid groups. In all cases the rate of reduction is much less in these spaces than in the developing leaves with their zoöids. There are, then, in this band of developing zoöids two quite distinct gradient patterns: first, the general decrease in rate of reduction in the developing zoöid groups from proximal to distal levels of the band and, second, the local decrease in rate from the developing zoöids of each group to the tissue between adjoining groups.

INDICATOR PATTERNS OF THE SCYPHISTOMA OF *Aurelia*

Budding, stolon development, and reconstitution of this scyphistoma and change of position by means of stolon attachment and contraction have been described by Gilchrist (1937) and are considered here only in their relation to gradient pattern. In the fully developed scyphistoma, a body or "hydranth" (Gilchrist's term) is usually distinguishable from the more slender stalk by its larger diameter, though the difference in diameter varies considerably. The manubrium, with the mouth at its center, is short and is surrounded by tentacles, which may be said to be "typically" sixteen but vary somewhat in number. Buds and stolons develop from different levels of the stalk, buds usually from the more distal levels, stolons nearer the base. Figure 29 is an outline of a scyphistoma in which the difference in diameter between distal body and stalk is greater than in some individuals. In this figure the reduction pattern of methylene blue and of Janus green is indicated by arrows. Reduction progresses basipetally in the tentacles when they are widely spread. In the higher dye concentrations the tentacles often contract and cover the manubrium more

or less completely, as indicated in Figure 30. In these cases the distal sides of the tentacles reduce earlier than the outer proximal sides, and usually with an acropetal, instead of the usual basipetal, decrease in rate. This is regarded as resulting merely from more rapid oxygen decrease in the confined space between the tentacles and the manubrium, the decrease being usually more rapid in the basal regions of the tentacles near the manubrium because the oxygen uptake may extend from the stalk into the proximal region of the body or hydranth, as indicated in Figure 29. Reduction is usually slightly more rapid in the middle region of the stalk and decreases again toward its base (Fig. 29). In this figure, two stolons are present, one arising from the base of the stalk, the other slightly above it. In the basal stolon the gradient is acropetal throughout the entire length; in the other the gradient is acropetal over most of the length, but at the distal



FIGS. 29–31.—Scyphistoma of *Aurelia*. Fig. 29, general gradient pattern. Fig. 30, contraction and approximation of tentacles, resulting in modification of reduction pattern by local more rapid oxygen decrease in confined space formed by tentacles. Fig. 31, early stolon bud.

of the manubrium, as well as that of the tentacles, plays a larger part in decreasing the oxygen in this space than at more distal levels of the tentacles. In detached tentacles the rate of reduction shows a distinct basipetal decrease except for a very short region immediately distal to the cut end, where the rate of reduction is increased by the injury. The manubrium reduces about as rapidly as the tentacles, and the reduction progresses basipetally over most of the body-length. The stalk, however, usually reduces somewhat more rapidly, and a short gradient, decreasing acropetally, end there is a short region in which reduction decreases basipetally. As the stolon elongates, its distal end sooner or later becomes slightly enlarged, with thicker ectoderm than in other parts. This region secretes a substance, apparently perisarcal in character and serving for attachment of the stolon to the substrate. After attachment, shortening of the stolon may occur, and change in position of the parent-individual or of a bud connected with the stolon results. Separation of buds from parents very commonly occurs in this way, as Gilchrist has shown. In Figure 29 the more

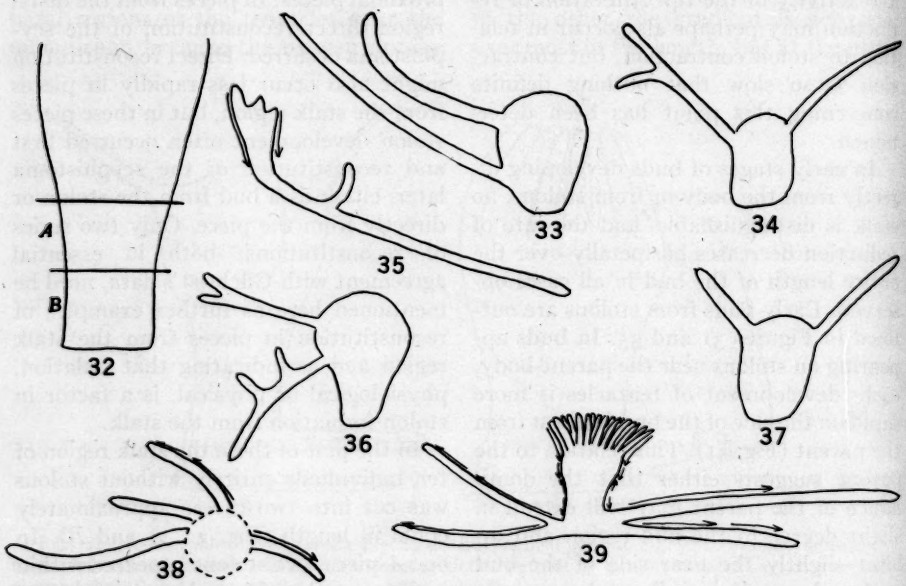
distal stolon, with its short basipetal gradient at the tip, has developed or is developing the terminal region of secretion and attachment, but in the more basal stolon there is no evidence of this region. The absence of any definite reduction gradient pattern in many stolons suggests that the stolon pattern varies with its activity in elongation and with the activity of the tip. Alteration of reduction may perhaps also occur in relation to stolon contraction, but contraction is so slow that nothing definite concerning this point has been determined.

In early stages of buds developing directly from the body or from stolons, no stalk is distinguishable, and the rate of reduction decreases basipetally over the entire length of the bud in all cases observed. Early buds from stolons are outlined in Figures 31 and 35. In buds appearing on stolons near the parent-body, early development of tentacles is more rapid on the side of the bud farthest from the parent (Fig. 31). This relation to the parent suggests either that the dominance of the parent may still extend in slight degree to the bud region and inhibit slightly the near side of the bud or that the gradient of the stolon extends into the bud. When two buds develop close together, as they may do on isolated pieces, tentacle development is more rapid on the sides of the buds farthest from each other than on the adjoining sides. This suggests slight mutual inhibition on the adjoining sides. The later appearance of the stalk, the higher rate of reduction in it than in more distal regions, and the development of buds and stolons from it, all seem to indicate a partial physiological isolation (Child, 1941, pp. 11, 82, and chap. ix) from the dominance of distal regions.

In connection with the differences between the hydranth or body and the stalk and the question of some degree of physiological isolation in the stalk, certain characteristics of reconstitution of physically isolated pieces are of interest. In pieces isolated from the different body-levels of the scyphistoma, Gilchrist (1937) found a decrease in rate and often in frequency of direct reconstitution of a scyphistoma from distal to proximal pieces. In pieces from the distal region direct reconstitution of the scyphistoma occurred. Direct reconstitution might also occur less rapidly in pieces from the stalk region, but in these pieces stolon development often occurred first and reconstitution of the scyphistoma later, either as a bud from the stolon or directly from the piece. Only two series of reconstitutions, both in essential agreement with Gilchrist's data, need be mentioned here as further examples of reconstitution in pieces from the stalk region and as indicating that isolation, physiological or physical, is a factor in stolon formation from the stalk.

In the first of these the stalk region of ten individuals entirely without stolons was cut into two pieces approximately equal in length (Fig. 32, *A* and *B*). In one *A*-piece, two stolons appeared within 24 hours and within 3 days five stolons, two of them branched, developed on three of the *A*-pieces (Figs. 33–35); reconstitution of a small scyphistoma was occurring in one of these (Fig. 35); in seven *A*-pieces there was no visible reconstitution. At the same time, two stolons each had developed in four of the *B*-pieces—a total of eight; six pieces were still without evidence of reconstitution; and there was no scyphistoma reconstitution in any. Figures 36 and 37 show 3-day stolons in two of the *B*-pieces. After 16 days there were stolons on five *A*-pieces, one piece with three, two with two each, and two with one each—a total of nine. Reconstitution

of a scyphistoma had occurred on seven pieces; one piece was alive but without development; and two pieces had been lost. At the same time there were stolons on eight *B*-pieces; four each on two pieces; three each on two pieces; two each on two pieces; and one each on two pieces—a total of twenty stolons. Scyphistoma reconstitution had occurred on basipetal decrease in rate, seen at the tips of some stolons, as noted above, was not seen in these stolons. The scyphistoma pattern was like that of the full-grown animal; the rate of reduction decreased basipetally in the tentacles and from the manubrium basipetally in the body, as distinguished from the stalk, but the increase in rate in the stalk appeared as in



FIGS. 32–39.—Stolon development in isolated stalk pieces. Fig. 32, *A*, *B*, levels of section of stalk. Figs. 33–35, stolons 3 days after section all in *A*-pieces. Figs. 36 and 37, stolons 3 days after section in two *B*-pieces. Figs. 38 and 39, stolons on two *B*-pieces 16 days after section; in Fig. 39 gradient pattern of reconstituted scyphistoma is indicated.

all ten of the *B*-pieces and without stolon formation on two pieces. Figures 38 and 39 show stolons in two of the *B*-pieces. In Figure 38 the stolons are viewed from above, and only the outline of the reconstituted scyphistoma is indicated in broken line. In Figure 39 the reconstituted organism is seen from the side. In Figures 38 and 39 the reduction patterns are indicated. The stolon patterns are all slightly acropetal or without distinct gradient. The short gradient with later stages. In the second series of reconstitutions twenty pieces, comprising approximately the entire stalk region, i.e., the region *A* and *B* of Figure 32 in a single piece, were cut from scyphistomas without stolons. Within 2 days one stolon had developed on each of thirteen pieces; two stolons on one piece; and none showed any indication of scyphistoma development. In both these series of physically isolated pieces of the stalk, stolon development is much more rapid

and extensive than in intact individuals. It appears highly probable that partial physiological isolation in the stalk of intact individuals and physical isolation of pieces of the stalk are factors in the development of stolons.

As in various other organisms, patterns of intracellular reoxidation following reduction depend on the oxygen available. If oxygen increase is slight or gradual after reduction, reoxidation gradients are opposite in direction to the reduction gradient. Apparently, oxygen becomes sufficient to permit reoxidation earlier in regions of less rapid, than in those of more rapid, oxygen uptake. With rapid and great oxygen increase or on removal to water or dye solution of high oxygen content, reoxidation usually progresses in the same directions as did the preceding reduction. In the first case oxygen available in relation to oxygen uptake of the different regions appears to determine rates of reoxidation; in the second case differences in oxidase activity apparently become the factor determining differences in rate of reoxidation.

DISCUSSION

In branching gymnoblast hydroids, e.g., *Pennaria*, *Tubularia*, and various other forms, the first hydranth formed becomes and remains the apical hydranth of the branching system; the bud developing at a certain distance below it becomes and remains the apical hydranth of a lateral branch. The axial system in these forms is monopodial, like the monopodial axes of many plants. In many branching calyptoblast hydroids the axial system is sympodial. In these the first hydranth remains apical only a short time in the growing organism. It is displaced to a lateral position by the first hydranth bud below it. This hydranth is again displaced from its temporary apical position by the next bud; the same sequence occurs in the branches. Instead of being the oldest, as in the monopodial systems, the apical hydranth of the sympodial systems is the youngest (Child, 1919, 1941, pp. 319–21; Hyman, 1940, pp. 404–5). Somewhat later it was found that, in the embryonic development of the gymnoblast *Corymorpha*, the primary gradient of the planula persisted, its apical or anterior end becoming the region of hydranth formation and remaining the high end of the gradient pattern of the organism throughout life (Child, 1926). In *Tubularia* and certain other gymnoblasts, primary polarity apparently persists. However, it was also determined that in the embryonic development of several calyptoblast hydroids the primary gradient pattern of the planula, with its high end anterior in swimming, did not persist but was gradually more or less completely replaced by a second gradient, appearing first at the posterior end and gradually extending anteriorly as the planula elongated. This second gradient was opposed in direction to the primary gradient, i.e., its high end was at the original posterior end of the planula. As the stage of free swimming came to an end, the planula became attached by the original anterior end, and the first hydranth developed at the original posterior end, the high end of the secondary gradient. The appearance of the second gradient was regarded as indicating a certain degree of physiological isolation from the dominance of the original anterior end, as the planula elongated and this dominance and the primany gradient became progressively weaker. In other words, the second gradient and the first hydranth developing from its high end were interpreted as representing the first bud in these calyptoblast hydroids, the only difference between this and later stages of hydroid development

being that this first bud was terminal and later buds were lateral (Child, 1925). Hyman (1940, pp. 405–7) notes that monopodial pattern prevails among gymnoblast hydroids but describes embryonic development of a calyptoblast hydroid with attachment of the planula by the original anterior end and does not raise the question whether a reversal of polarity occurs in development of gymnoblasts (pp. 434–35). However, it seems probable, in the light of the incomplete data at hand, that in the embryonic development of gymnoblast hydroids with monopodial axes there is no such reversal of polarity as in the planulae of calyptoblast forms with sympodial axes in the hydroid. It has seemed advisable to call attention to these points concerning hydroid development in order to bring the *Polyorchis* planula into its proper relation to other forms.

The medusa *Polyorchis* is a calyptoblast, and its planulae provide another example of reversal of gradient pattern in this group. The primary planula gradient pattern is that of the later oöcyte, cleavage, and blastula stages and apparently originates in the gonad in relation to the position of the oöcyte nucleus near the free pole of the cell. It has not been possible thus far to observe the development of the hydroid from the planula under laboratory conditions, but the appearance of the secondary gradient at the posterior end of the planula and the gradual reversal of gradient pattern and of polarity in the planula have been conclusively demonstrated. Many planulae do become attached by one end after the reversal of polarity. This end is often clearly distinguishable as the original anterior end, i.e., the low end of the secondary gradient, but some planulae become so long and slender that it is not certain which end becomes attached. However, it appears improbable, in the light of the earlier data on calyptoblast development, that the high end of the secondary gradient becomes the region of attachment in any case.

The early development of the Pennatulacean, *Stylatula*, presents several features of interest as regards gradient pattern. The position of the oöcyte nucleus close to the stalk instead of near the free pole of the cell is unusual. In oöcytes of various other organisms it has been observed that intracellular oxidation and reduction of indicators are more rapid about the nucleus or particularly where the nucleus is nearest the surface of the cell and decrease from this region, but in these cases the nucleus has been nearer the free, than the attached, pole. Intracellular reduction has been observed in large numbers of oöcytes of *Stylatula* with a wide range of dye concentrations and staining periods in order to decrease as far as possible the danger of error, but the results have been consistent. It appears somewhat improbable that the growing oöcyte obtains more oxygen from the region nearest the stalk than from elsewhere. The oxygen supply seems to be a factor in determining the position of the oöcyte nucleus in certain other forms. Is it possible that the oxygen supply is adequate in all parts of the cell and that the supply of material for growth is largely from the stalk and determines the nuclear position? The full-grown egg cell contains a large amount of yolk and is almost opaque. Possibly a differential entrance of material for growth is concerned in determining nuclear position. The data as presented are believed to be correct, but the question of the significance of the nuclear position and of the reduction gradient in the oöcyte remains unanswered.

In the development of a solid blastula and the formation of entoderm by delamination and further division, *Stylatula*

and alcyonarians generally differ from many other coelenterates. The slightly thicker ectoderm about one pole—the posterior pole in swimming—in the spherical preplanula stage which follows the blastula is the region of least rapid reduction in the primary gradient. However, in *Stylatula*, as in *Polyorchis*, a second gradient appears at the original posterior end of the planula as it elongates and gradually replaces more or less completely the original gradient; its high end becomes the oral end of the primary polyp, a reversal of gradient pattern and of polarity, involving most, and perhaps finally all, of the body. The development of the oral end from the end of the alcyonarian planula which is posterior during the stage of active swimming has been known for many years. Earlier literature on alcyonarian development has been cited by Mathews (1916).

The appearance of the second gradient in planulae of calyptoblast hydroids and in this alcyonarian is regarded as essentially an agamic reproduction—a bud, resulting from a certain degree of physiological isolation of the region of the planula originally posterior as elongation of the planula body occurs. In the alcyonarian the original anterior end of the planula becomes the stalk of later stages. The original posterior end—the region of least rapid intracellular reduction in the primary gradient—becomes secondarily the region of the highest rate of reduction in the organism—the oral end and the dominant region of the primary polyp as the primary gradient decreases and is finally almost or quite obliterated. It is a point of some interest that the effective ciliary beat and the orientation in swimming are not reversed as the new gradient pattern appears. However, swimming activity decreases and is replaced by contractile activity, often more or less peristaltic in character. The polyp appears to be approaching the stage when, under natural conditions, it would begin burrowing into the mud.

In the localization of the first zoöid bud on the primary polyp, *Stylatula* resembles *Renilla* (Wilson, 1883). The appearance of this bud only a short distance from the oral end suggests, as does the later development of numerous zoöids, that the dominance of the oral region is effective only over short distances. The bud represents the appearance of another new gradient pattern. Further development of buds was not observed in the laboratory. In the mature organism zoöids appear in transverse groups "leaves" along the main axis.

The pinnate tentacle and the reversal of the tentacle gradient associated with the order of development of the pinnules constitute another point of difference between *Stylatula* and probably alcyonarians generally and other coelenterates with unbranched tentacles. It also suggests a very short range of dominance in the developing tentacles. In the unbranched coelenterate, tentacle rates of primary intracellular oxidation and of reduction, preceding injury by the indicator, decrease basipetally throughout life.

The high rate of dye reduction in the zoöid groups of the bands of developing zoöids and the decrease in rate from proximal to distal levels of the band as development of the zoöids progresses are what might be expected from observations on gradient patterns in buds of other forms. The bud shows a gradient pattern of its own in its earliest stages, and, as its development progresses, its rate of reduction decreases. Also the developing zoöid buds and leaves are undoubtedly regions of greater oxidative

activity than are the spaces between the groups.

One point concerning the oöcytes and eggs of *Polyorchis* and *Stylatula* deserves particular attention. In both, the region of most rapid intracellular oxidation and reduction does not move with the nucleus to the center of the egg following fertilization and the change in nuclear position before the first cleavage. From the observations on gradient pattern in these eggs, it appears that the indicator gradient pattern is not essentially altered by the change in nuclear position but becomes the primary polar gradient of the organism. It is suggested that the apparent independence of nuclear position and gradient pattern following fertilization is probably associated in some way with the cessation of growth of the oöcyte. After it is full grown and separated from the parent-body, the egg is no longer taking in and transforming food materials into protoplasm or yolk. The relation between nucleus and cytoplasm is undoubtedly different from that characteristic of the growth period. However, it seems evident that the gradient pattern impressed upon the cell during the growth period of the oöcyte persists without relation to later nuclear position. In both these forms the evidence indicates that it persists until it is displaced and more or less completely obliterated by the appearance of the secondary gradient and the reversal of polarity.

The scyphistoma of *Aurelia* resembles other coelenterates in showing a decrease in rate of intracellular reduction basipetally in the tentacles and the body as distinguished from the stalk. As already noted, the secondary development of the stalk, the higher rate of reduction in it than in the body distal to it, the development of buds and stolons from it while it is still a part of the intact animal, and the more rapid development of stolons in physically isolated pieces all suggest that it is a region partially physiologically isolated from the dominance of the intact organism. The scyphistoma stolon differs from the stolons of most coelenterates in functioning not only as a source of scyphistoma buds but, by attachment of its tip and shortening, as a means of separating buds from the parent-body or even changing the position of more advanced individuals. Its gradient pattern, when a distinct pattern is present, also differs from that of the stolons of other coelenterate groups which grow at the tip and have a gradient decreasing basipetally.

SUMMARY

1. In the later oöcyte of the calyptoblast medusa *Polyorchis*, the nucleus is near the free pole of the cell and the oxidation-reduction indicator gradient decreases from the region about the nucleus.

2. This gradient becomes the primary polar gradient and persists into the earlier planula stages, its high end being the anterior end in swimming. As the planula elongates, a second gradient, decreasing from the original posterior end, appears and extends anteriorly along the planula. Hydroid development from the planula has not been observed in the laboratory, but attachment by the original anterior end has occurred in many individuals. The second gradient is regarded as representing the first bud and as resulting from increasing physiological isolation in the elongating planula. It probably becomes the primary hydroid gradient, as in other calyptoblast hydroids whose development is known. There is in these forms a reversal of gradient pattern and of polarity during elongation of the planula.

3. In the growing oöcyte of the pen-

natulid *S. elongata*, the nucleus is situated near the stalk of the entodermal membrane, which surrounds the cell, and intracellular reduction decreases in rate from a zone about the nucleus. This gradient pattern persists into the earlier planula stages, its high end being anterior in swimming; but in this form also, as the planula elongates, a second gradient appears, with the rate of intracellular reduction decreasing from the original posterior end. This becomes the gradient of the primary polyp, and its high end, the original posterior end of the planula, becomes the oral end and the region of most rapid reduction in the primary polyp. Here, as in the calyptoblast *Polyorchis*, there is a reversal of gradient pattern and of polarity, and here also this reversal is regarded as the first bud, resulting from the increasing physiological isolation of the original posterior end as the planula elongates and the primary gradient gradually decreases.

The rates of intracellular reduction of indicators decrease from the tentacle tips and the manubrium through the body or hydranth region, as distinguished from the stalk, in the full-grown nonproliferating scyphistoma of *A. lobata* but become more rapid in the stalk. In earlier bud stages the stalk is not present, and the gradient decreases basipetally throughout. The secondary development of the stalk, the more rapid rate of reduction in it, the development from it of scyphistoma buds and of stolons capable of giving rise to scyphistoma buds, and the increase in development of stolons in physically isolated pieces suggest that it is a region partially physiologically isolated from the dominance of more distal regions of the scyphistoma. The rate of reduction in the stolon usually decreases acropetally, except for the short region at the tip, the region of attachment, where there is in some stolons a short gradient, decreasing basipetally. In other stolons this terminal region of attachment has apparently not yet developed, and in still others no distinct gradient pattern is distinguishable.

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THE SITE OF THE INHIBITORY ACTION OF ETHYL CARBAMATE AND SODIUM AZIDE ON THE OXYGEN UPTAKE OF EMBRYONIC CELLS[1]

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THE inhibitory action of such reagents as ethyl carbamate and sodium azide on the O_2 uptake of various biological forms seems well established (McElroy, 1947; Hollinger, Fuhrman, Lewis, and Field II, 1949; Bodine, 1950*a*, *b*). The manner in which these effects are produced, however, is not as yet entirely clear and, from the standpoint of their basic cellular action, deserves much further consideration (Cross, Toggart, Covo, and Green, 1949; Loomis and Lipmann, 1949). Previous reports have indicated that cellular responses to these reagents are in many instances conditioned not only by the specific biological forms employed but also by the physiological states of the materials themselves (Clifton, 1946). The present investigation was undertaken to determine the effects of ethyl carbamate and sodium azide on the O_2 uptake not only of the intact embryonic organism but also of the intact embryonic cell and various intracellular constituents, such as nucleus, mitochondria, cytoplasm, etc. By means of fractional centrifugation techniques, the various constituents of the normal embryonic cell seem rather easily separated for experimental studies, and in the case of the isolated embryo of the grasshopper, *Melanoplus differentialis*, employed in the present investigation, such techniques have proved quite adequate (Bodine, 1950*a*, *b;* Bodine and Lu, 1950*a*, *b*).

[1] Aided by a grant from the National Institutes of Health. Acknowledgment is gratefully made to Etta Andrews for technical assistance in carrying out this investigation.

MATERIAL AND METHODS

Embryos of the grasshopper, *M. differentialis*, were dissected from eggs of known history and treated as previously noted (Bodine and Boell, 1936). One hundred embryos per milliliter of suspension medium [0.9 per cent, phosphate-buffered (pH 6.8) sodium chloride] were used, and homogenates, nuclei, or cytoplasmic constituents were prepared in this medium and standardized on this basis. The homogenization, centrifuging, washing, etc., of materials were essentially similar to those already described (Bodine, 1950*a*, *b;* Bodine and Lu, 1950*a*, *b*). C.P. solutions of all reagents were made up in phosphate-buffered 0.9 per cent NaCl, and the concentrations shown are the final ones representing those in the respiration flasks. Oxygen determinations, unless otherwise indicated, were carried out with standard Warburg manometers at 25° C., by means of respiration flasks of 5-ml. capacity. Controls were run for 30–60 minutes before the addition of reagents, and experiments ran for 2–2½ hours. A minimum of twelve to fourteen manometers was run for each concentration of reagents. Further details of individual experiments are indicated below in explanations of figures.

Ethyl carbamate.—The inhibitory effects of ethyl carbamate (E.C.) on the

endogenous O_2 uptake of intact embryos, homogenates, precipitate (nuclei), and supernatant (mitochondria and microsomes) are graphically summarized in Figure 1. An examination of this figure shows that a marked and strikingly similar inhibition of O_2 uptake is found for all

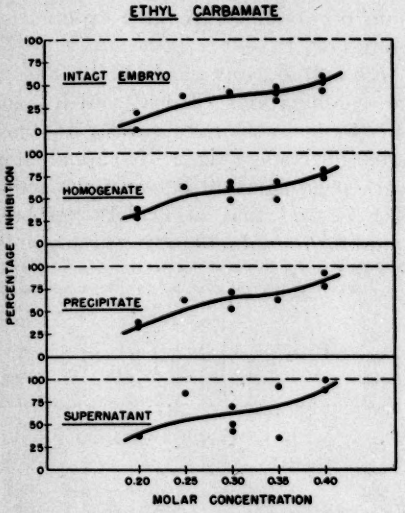

FIG. 1.—Shows effect of E.C. on the O_2 uptake of intact embryos, homogenate, precipitate (washed nuclei) and supernatant (mitochondria and microsomes). Data expressed as per cent of normal in 0.9 per cent NaCl suspension medium taken as 100 per cent. One hundred intact embryos per cubic centimeter of suspension medium; 1 cc. of homogenate equal to 100 embryos. Homogenization carried out at room temperature (22°–24° C.). Equilibration 15 minutes. Readings every 10 minutes. Values given are averages for 70-minute exposure to reagent and represent readings from a minimum of twelve manometers for each concentration of reagent. Centrifugation carried out at 600 × gravity for nuclei; 1,200 × gravity for mitochondria; 1,800 × gravity for microsomes. Washed intact nuclei contain inherent enzyme-substrate system. Curves drawn through points by inspection.

conditions of the materials employed. It would thus seem that the living embryonic cell, whether intact or its isolated parts, are susceptible to the inhibitory effect of E.C. Structure or intactness of the cell or embryo is apparently not essential for its inhibitory action on O_2 uptake.

Sodium azide.—It has previously been shown that sodium azide (NaN_3) produces marked inhibition of the O_2 uptake of the actively developing intact grasshopper embryo (Bodine, 1950*b*). Present results confirm these observations and also show, in addition, that the various cellular constituents behave toward this reagent in a strikingly similar fashion. The results for the effect of different concentrations of the reagent on the O_2 uptake of the embryo and intracellular constituents are graphically summarized in Figure 2.

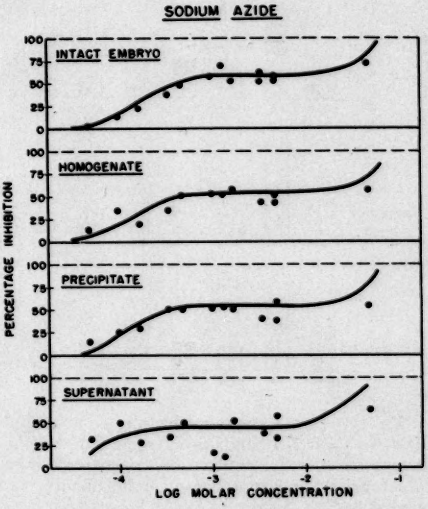

FIG. 2.—Same as Fig. 1, except for NaN_3

NaN_3, methylene blue, and 2,4-dinitrophenol.—Previous reports have indicated a marked difference in the response of the intact grasshopper embryo treated with E.C. or NaN_3 to methylene blue (M.B.) and 2,4-dinitrophenol (2,4-D.N.P.) (Bodine, 1950*a*, *b*). A striking antagonism between M.B. and these reagents seems to exist, while for 2,4-D.N.P. no such

phenomenon is observed (Bodine, 1950). Since these observations were made upon intact embryos, it becomes of some further interest to compare these results with those found for the various intracellular constituents. The results of such experiments are graphically summarized in Figure 3. An inspection of this figure shows that, for intact embryos, M.B. antagonizes to a marked degree the inhibitory action of NaN_3, while D.N.P. has little, if any, effect. Homogenates made from embryos show strikingly similar results. Washed intact nuclei from the cells of these embryos react in a similar fashion. Granules (mitochondria and microsomes) normally react little or not at all to M.B. and to D.N.P. (Bodine and Lu, 1950*b*). However, when treated with inhibitory doses of NaN_3, additions of M.B. show some antagonistic action. No similar reactions to D.N.P., however, are noted. The lack of a marked stimulation of O_2 uptake of mitochondria and microsomes by M.B. when suspended in normal physiological medium and its antagonistic action for NaN_3 when the same material is subjected to it are at present rather difficult to explain. No such reactions occur for comparable experiments using D.N.P.

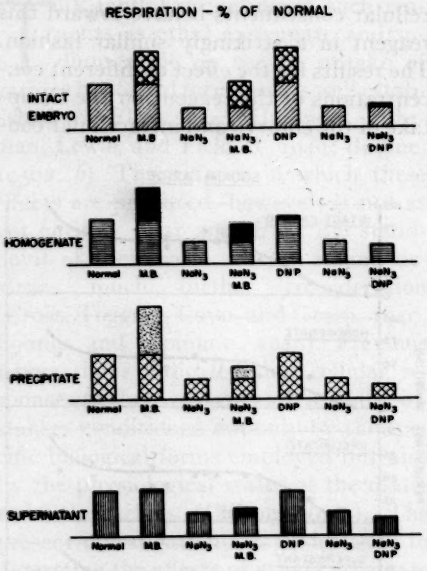

FIG. 3.—Shows relative effects of combinations of M.B., 2,4-D.N.P. and NaN_3 (1.66×10^{-3} M) on O_2 uptake of intact embryo, homogenate, precipitate (nuclei), and supernatant (mitochondria and microsomes).

E.C., M.B., and 2,4-D.N.P.—Results for combinations of E.C., M.B., and D.N.P. are strikingly similar to those noted for NaN_3 (Fig. 4). No apparent or marked antagonism between the action of E.C. and that of D.N.P. has been found for the intact embryo or any of its constituent parts. It would thus seem that other modes of action than those for M.B. must exist for D.N.P. Intactness of the cell seems most essential for the primary stimulating action of D.N.P., as previously pointed out and as confirmed in the results of the present experiments (Bodine and Lu, 1950*b*).

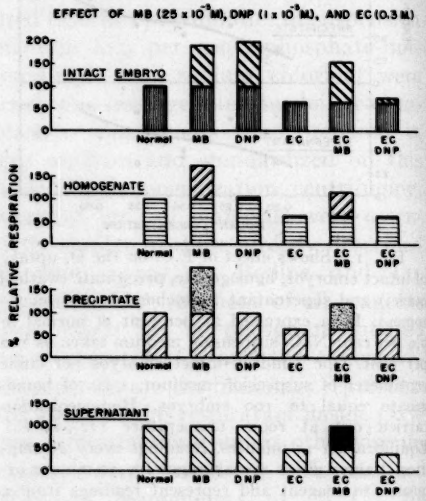

FIG. 4.—Same as Fig. 3 except for E.C. Note antagonistic action of M.B. and practically no such effect for 2,4-D.N.P.

Experiments resembling those above, but carried out on the intact and homogenized nuclei, show rather striking and more or less similar results (Fig. 5). M.B. markedly stimulates the O_2 uptake of the intact nucleus but has little, if any, effect upon the homogenized ones (Fig. 5). On the other hand, D.N.P. does not stimulate the O_2 uptake of either intact or homogenized nuclei. It would thus seem that the integrity of the entire cell is essential for any stimulating action of D.N.P. Moreover, M.B. is apparently most active in its stimulating effects when concentrated on or at the surface of the intact nucleus, since, when nuclear structure is destroyed, no such stimulating action is evident (Fig. 5). On the other hand, E.C. and NaN_3 seem effective in producing their inhibitory action, irrespective of the nature of the living material, i.e., whether intact or homogenized, and it appears that no specific cell structure as such is necessary for their action.

SUMMARY

1. The inhibitory action of E.C. and NaN_3 on the O_2 uptake of intact grasshopper embryos (*M. differentialis*), their homogenates, and intracellular constituents has been studied.

2. Both reagents produce similar inhibitory action on the O_2 uptake of intact embryos, homogenates, nuclei, mitochondria, and microsomes.

3. Intactness of the organism or cell does not seem essential for the inhibitory action of these reagents.

4. The antagonistic action of M.B. for these reagents occurs in the intact embryo, homogenate, and intracellular constituents.

5. 2,4-D.N.P. shows no antagonism for the reagents and exerts its stimulating action only on the intact embryo.

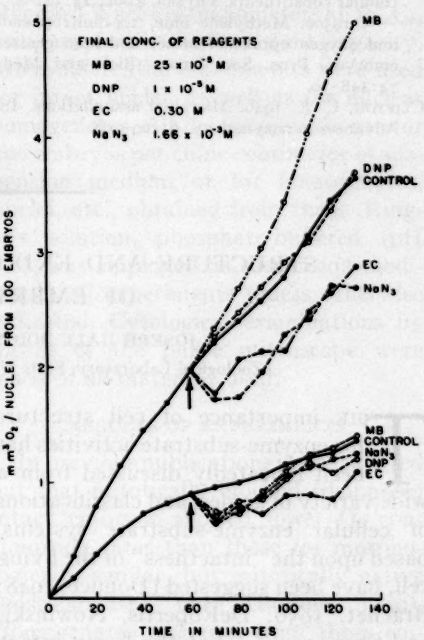

FIG. 5.—Shows effect of reagents on O_2 uptake of washed *intact* nuclei (*upper curves*) and homogenates made from them (*lower set of curves*). Arrow indicates time of introduction of reagent into respiration chamber. *Ordinate*, cubic millimeters of O_2 for nuclei from 100 embryos per cubic centimeter; *abscissa*, time in minutes. Concentration of reagents noted. A microdifferential manometer was employed for all experiments with nuclei.

6. M.B. stimulates the O_2 uptake of the intact washed nuclei but has no action on their homogenates.

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STRUCTURE AND ENDOGENOUS OXYGEN UPTAKE OF EMBRYONIC CELLS[1]

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THE importance of cell structure for enzyme-substrate activities has been repeatedly discussed from a wide variety of angles, and classifications of cellular enzyme-substrate systems, based upon the "intactness" of the living cell, have been suggested (Donuce, 1948; Brachet, 1950; DeRobertis, Nowinski, and Saez, 1950). That the living intact cell functions as a co-ordinated physicochemical system is well established. Changes in the enzyme-substrate activities brought about by the rupture or breaking-down of the intact cell have been variously reported (Dounce, 1948; Brachet, 1950; DeRobertis, Nowinski, and Saez, 1950). Tissue slices and sections have been extensively employed in obtaining data concerning the relations of enzyme substrates and cell structure (Claude, 1940; Hogeboom, Claude, and Hotchkiss, 1946; Schneider, 1946; Schneider, Claude, and Hogeboom, 1948). Liver, kidney, and brain of vertebrates and especially of mammals have been extensively used, and considerable data are at hand for these organs.

Little data, however, seem available for invertebrates, exclusive of the ova of marine forms like the sea urchin. Recent investigations on the ova of the sea urchin have been concerned largely with cell-free particulate systems and not with homogenates obtained directly from the ova themselves (Clowes, Keltch, Strettmatter, and Walters, 1950). The embryo of the grasshopper (*Melanoplus differentialis*), dissected from the egg and freed from yolk, has been shown to be extremely suitable biological material for analyses of the parts played by cell structure and respiratory enzymes, since O_2 consumption can be readily correlated with the cytological structure and developmental hystory of the embryo (Bodine, 1950). By using the intact embryo, free of yolk and of known developmental history, one readily obtains reproducible re-

[1] Aided by a grant from the National Institutes of Health. Acknowledgment is gratefully made to Etta Andrews for technical assistance in carrying out these experiments and to William West for photomicrographs.

sults from which rather definite standards may be formulated (Bodine and Lu, 1950).

The present paper deals with extensive data on the O_2 uptake of intact embryos and their homogenates and intracellular constituents, as well as the quantitative relations existing between them. No extraneous substrates have been employed, and the O_2 consumption measured represents that from enzyme-substrate systems inherent in the living material.

MATERIAL AND METHODS

Embryos of the grasshopper, *M. differentialis*, were dissected from eggs of known developmental history and prepared as previously indicated (Bodine and Lu, 1950). Homogenization was carried out by means of a motor-driven glass plunger (Bodine and Lu, 1950.) For long homogenization periods, the temperature was maintained by immersion of tubes in constant-temperature baths, while all others were carried out at room temperatures (22°–23° C.). Homogenates of embryos thus prepared contained intact and broken nuclei, mitochondria, microsomes, and other intracellular substances. Intact isolated nuclei are broken only with considerably more effort than that used for the production of the homogenate of the intact embryo. Two methods, in general, were employed. First, washed nuclei were frozen by exposure to dry ice (−20° C.) for a period of 5 minutes. They were next thawed at room temperature. This procedure was repeated three times. The nuclei were then homogenized for 10 minutes. All were completely broken, as confirmed by cytological examination (Pl. I, Fig. E). Second, washed nuclei were homogenized in a close-fitting glass homogenizer for 10–12 minutes at constant temperature (20° C.). No intact nuclei were present after such treatment (Pl. I, Fig. D). The O_2 uptake of nuclear homogenates apparently depends to a marked degree upon the method of preparation, as is shown in what follows.

The O_2 consumption was measured by standard Warburg manometers at 25° C., using respiration flasks of 5-ml. capacity. Microdifferential manometers were used for intact nuclei, as well as for nuclear homogenates. All determinations are for 100 embryos per cubic centimeter of suspension medium or for homogenates, nuclei, etc., obtained from them. Ringer's solution, phosphate-buffered (pH 6.8), was employed as suspension medium in all experiments unless otherwise indicated. Cytological examinations by means of the phase microscope were made of all materials used.

RESULTS OF EXPERIMENTS

In previous publications it was pointed out that mitotically blocked (diapause) intact embryos showed lower O_2 consumption rates than those for morphologically similar, but mitotically active (postdiapause), ones (Bodine, 1950). Homogenates prepared from these embryos, in general, have the same relative differences in O_2 consumption (Bodine and Lu, 1950). Additional data for postdiapause embryos and homogenates obtained in the present experiments are graphically shown in Figure 1. An inspection of this figure shows, as previously indicated, that about 60–70 per cent of the total O_2 uptake seems lost when intact embryos are homogenized, while some 30–40 per cent remains in the homogenate. These relative percentage differences between the O_2 uptake of intact and homogenized embryos remain extremely constant for all experiments thus far carried out. Washed intact nuclei consume approximately 50 per cent of the O_2 uptake of the embryo

homogenate from which they are obtained (Bodine and Lu, 1950). This value has been confirmed by further experiments in the present series and is indicated graphically in Figure 1. The O_2 uptake of homogenates prepared from washed intact nuclei depends to a great degree upon the method of their preparation, as shown below.

Nuclei frozen with dry ice (−20° C.).—Intact embryos subjected to dry ice and frozen have O_2 consumption rates no longer comparable to those for nonfrozen ones (see Fig. 2). Subjection of the intact embryo to dry ice not only destroys the permeability of cell membranes but also enzyme substrates as well, since, when such preparations are treated with methylene blue (M.B.) or 2,4-dinitrophenol (D.N.P.), no stimulating actions of their O_2 consumption, resembling those for nontreated intact embryos, are produced (Fig. 2). Frozen embryos become soft and sticky and lose all the morphological appearance of the nontreated ones. The O_2 uptake of the frozen embryos, as well as their homogenates, nuclei, and intracellular constituents, is greatly reduced when the intact embryo is first subjected to freezing with dry ice (Fig. 2). Washed intact nuclei from normal embryos, when frozen with dry ice, produce results similar to those when the intact embryo is frozen and the nuclei then separated by fractional centrifugation.

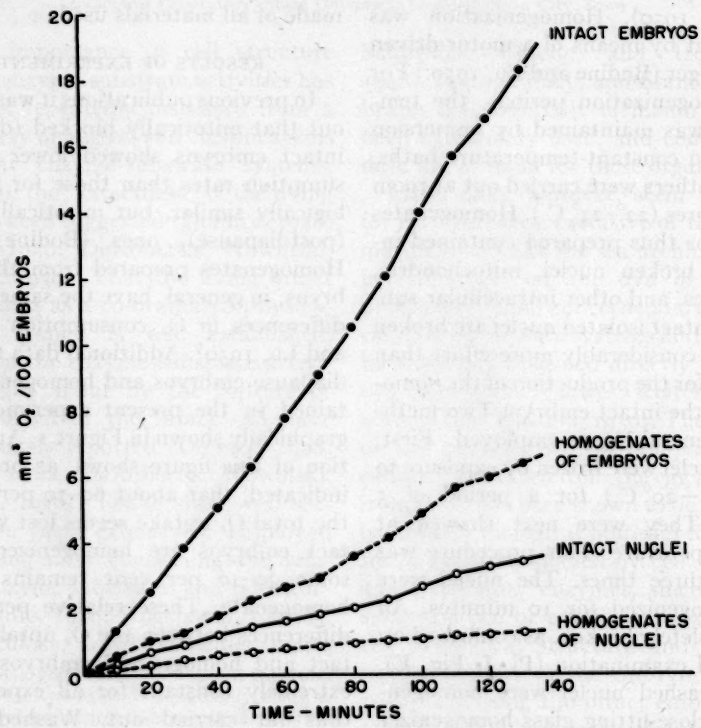

FIG. 1.—Shows O_2 uptake for intact postdiapause embryos and their homogenates, as well as for intact nuclei and their homogenates. For further description see text.

Washed intact nuclei homogenized without freezing.—Washed intact nuclei homogenized for periods of 10–12 minutes at 20° C. give O_2 consumption rates approximately 30–40 per cent of those for the intact nuclei from which they were prepared (Figs. 1, 2, and 3). It is of some interest to note that destruction of the intact nucleus results in a reduction of O_2 uptake (60–70 per cent) comparable to that for intact embryos and their homogenates. The O_2 uptake of nuclear homogenates prepared by the above method is not stimulated by the addition of M.B. or D.N.P. (Fig. 3), thus confirming data previously presented (Bodine and Lu, 1950).

Cytological studies have been carried out on all embryos and homogenates used, and phase microscope photographs of intact cells and intact and broken nuclei, along with supernatants, are shown in Plate I.

DISCUSSION

It has previously been pointed out and again confirmed that for the intact grasshopper embryo a reduction of some 60–

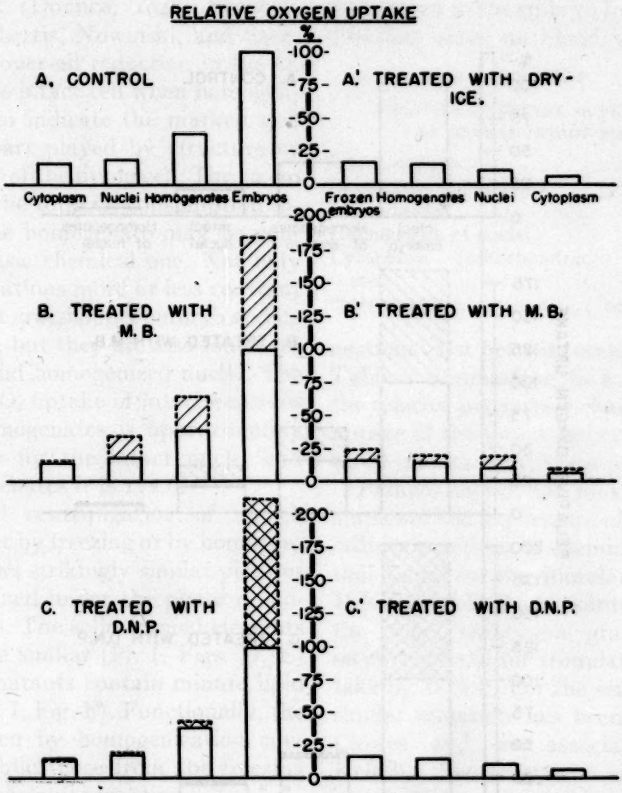

FIG. 2.—*A*, shows relative O_2 uptake for normal intact postdiapause embryos (100 per cent) and their homogenates and intracellular constituents, as well as O_2 uptake for embryos frozen in dry ice. *B*, effect of M.B. (25×10^{-5} M) on O_2 uptake of normal and frozen embryos, etc. *C*, same as *B*, except for D.N.P. (1×10^{-5} M). Cross-hatched areas represent increases in O_2 uptake due to addition of reagents.

70 per cent in O_2 consumption occurs as the result of homogenization (Bodine and Lu, 1950). The actual value of the O_2 uptake of the homogenates in these cases is apparently dependent upon the initial rate of O_2 uptake of the intact embryo, since homogenates made from embryos with actively growing and dividing cells (postdiapause stages) have O_2 consumption rates higher than those from embryos blocked or mitotically inactive (diapause stage) (Bodine, 1950). Apparently, basic chemical differences in the two types of embryos exist, and these differences, in turn, are present to the same relative degree in their respective homogenates. In the present experiments a similar relation has also been found for intact nuclei and their homogenates (Fig. 1). Curiously, the reduction in the rate of O_2 uptake of the nuclear homogenates, as compared with the intact nuclei from which they came, is similar to that found for the intact embryo and its homogenate, viz., 60–70 per cent. In the preparation of homogenates in general, structure or "intactness" is greatly interferred with, while the endogenous enzyme-substrate relations are also disturbed or displaced but not necessarily

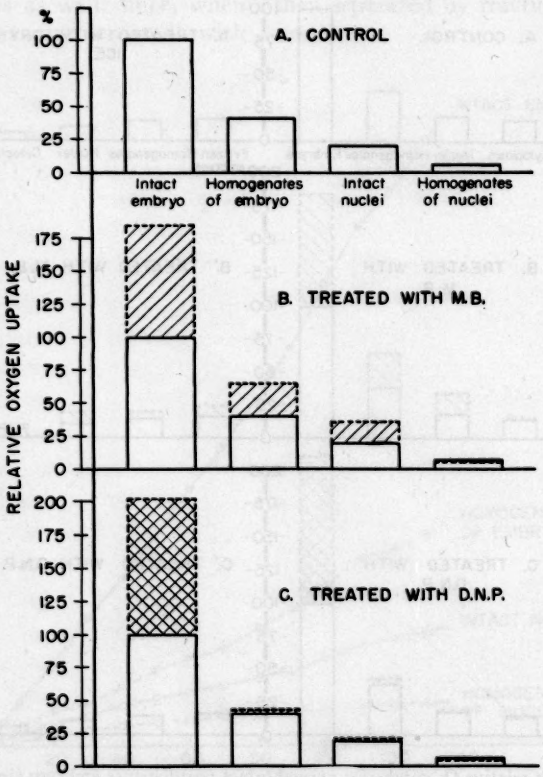

FIG. 3.—*A*, shows relative O_2 uptake of intact postdiapause embryos and their homogenates, intact nuclei and their homogenates. *B*, effect of M.B. on same. *C*, effect of D.N.P. on same. Cross-hatched areas represent increases in O_2 uptake due to additions of reagents.

destroyed. Intactness of the cell membrane for the stimulating action of D.N.P. seems essential, while similar conditions of the nuclear membrane are equally important for the stimulating action of M.B. (Bodine and Lu, 1950). Not only do intact embryos or nuclei, subjected to dry ice (−20° C.), suffer irreversible changes in permeability, but their enzyme substrates are also denatured or irreversibly affected (Fig. 2).

Data obtained from tissues such as liver in comparable situations tend to show reduction of O_2 consumption of their homogenates of approximately 60–70 per cent (Dounce, 1948; Brachet, 1950; DeRobertis, Nowinski, and Saez, 1950). This over-all reduction in the O_2 uptake of the intact cell when homogenized seems to indicate the marked and significant part played by structure or "intactness" of the living cell. The 30–40 per cent of the total O_2 consumption inherent in the homogenate may be considered a basic chemical one. Not only are these relations more or less constant for the intact grasshopper embryo and its homogenate, but they are also found for the intact and homogenized nuclei. The ratio of the O_2 uptake of intact embryos to their homogenates is approximately 1:2.9, while for the intact nuclei and their homogenates it is 1:2.6.

Fractional centrifugation of nuclei, broken either by freezing or by homogenization, shows strikingly similar pictures when examined under the phase microscope (Pl. I). The solid formed elements appear quite similar (Pl. I, Figs. D, E) while supernatants contain minute lipid globules (Pl. I, Fig. F). Functionally, the nuclei broken by homogenization consume O_2, while those from the freezing techniques are quite inactive.

It is of interest to note that in the present experiments no substrates or enzymes other than those inherent in the living embryos have been employed. No yolk or other extraneous materials from the egg are used, since embryos can be easily obtained free of such substances. Separation of the embryo from the yolk is of great importance in respiration studies on embryonic cells, since the addition of but small quantities of such materials greatly interferes with results normally obtained and variations in O_2 uptake become so great that consistent data are practically impossible to obtain. In the development of the embryo of the grasshopper, food substances are presumably elaborated in the yolk and then transferred to the embryo by processes of diffusion, since no blood vascular connections exist between embryo and yolk. Table 1 summarizes, in a general way, the relative percentage changes in the O_2 uptake of the intact embryo when separated into its constituent parts.

TABLE 1

RELATIVE O_2 UPTAKE AS PERCENTAGE OF NORMAL INTACT EMBRYO

Intact embryo	100		
Homogenate of embryo	30–35	(100)	
Intact nuclei	15	(50)	(100)
Homogenate of nuclei	5		(30)
Cytoplasm (mitochondria, etc.)	15	(50)	
"Structure"	60–70		

Perhaps one of the most striking examples of the importance of structure for cellular reactions to chemical reagents is that found for the stimulating action of D.N.P. and M.B. As pointed out above, the intact embryonic grasshopper cell seems essential for stimulation of O_2 uptake by D.N.P. For the sea-urchin egg a similar situation has been reported by Clowes and his associates (Clowes, Keltch, Strittmatter, and Walters, 1950). M.B. seems to exert its stimulating action in the grasshopper cell only when the nuclear membrane remains intact. Both these chemical reagents, how-

ever, produce similar qualitative results for mitotically blocked or active embryonic cells.

SUMMARY

1. The O_2 uptake of intact grasshopper (*M. differentialis*) embryos as well as of their homogenates has been determined.

2. The O_2 uptake of intact and homogenized nuclei from embryonic cells of grasshopper embryos has been determined.

3. Homogenization of intact embryos as well as of intact nuclei results in a loss of O_2 uptake of approximately 60–70 per cent. This reduction seems dependent on the *structure* or "intactness" of the living embryonic cell.

4. The O_2 uptake of homogenates, either of embryos or of nuclei, is 30–40 per cent of that for the intact structure.

5. The importance of structure for the endogenous O_2 uptake of living embryonic cells is pointed out.

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PLATE I

FIG. A.—Intact cells, from embryo smear on slide. Phase microscope, 970×.

FIG. B.—Intact nuclei. Homogenate of embryo subjected to 600×G. for 10 minutes. 430×. Intact nuclei sedimented.

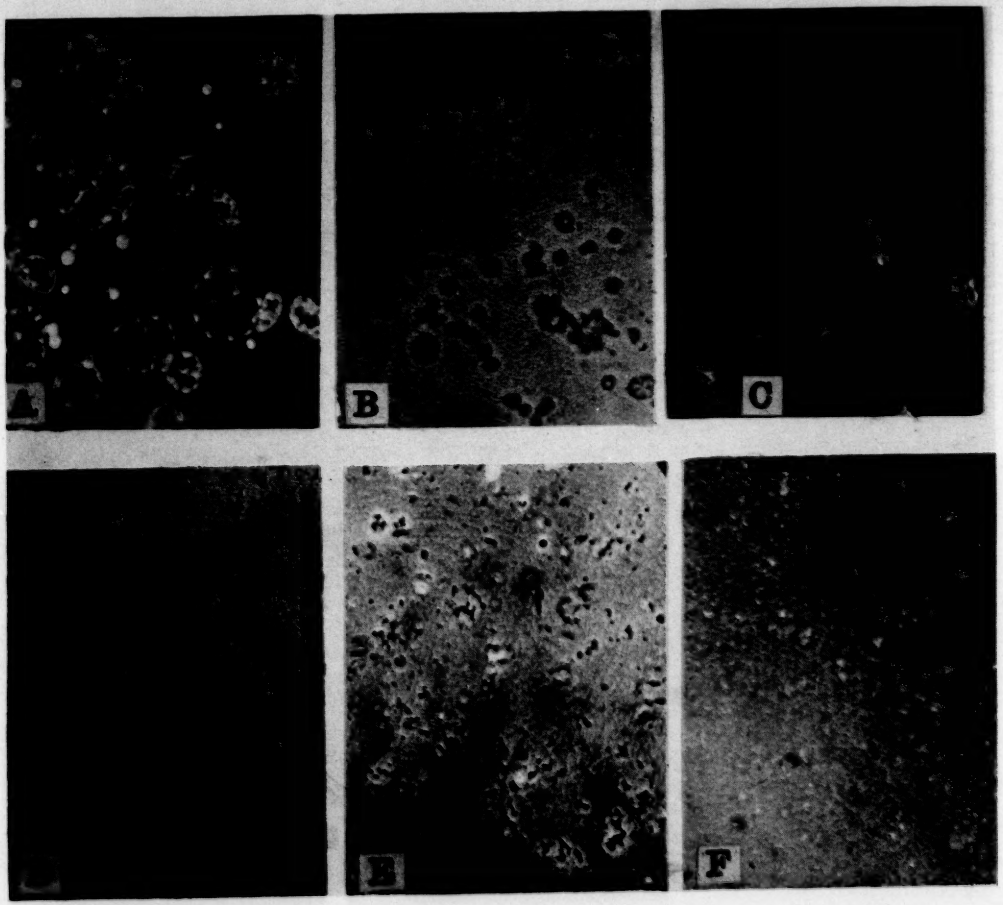
FIG. C.—Same as *B*, but with phase microscope.

FIG. D.—Broken nuclei. Washed intact nuclei subjected to homogenization by Potter-Elvehjem method. Phase microscope, 430×.

FIG. E.—Same as *D*, except nuclei broken by exposure to dry ice.

FIG. F.—Supernatant fraction from broken nuclei. Broken nuclei centrifuged at 1,800×G. for 2 minutes. Smaller granules and fat globules remain in supernatant fraction. Phase microscope, 970×.

PLATE I



A COMPARISON OF THE CHOLINESTERASE IN THE HEADS OF THE HOUSE FLY, THE COCKROACH, AND THE HONEY BEE

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Bureau of Entomology and Plant Quarantine, Beltsville, Maryland

It was observed by Richards and Cutkomp (1945) that acetyl-β-methylcholine was hydrolyzed nearly three times as fast as acetylcholine by the cholinesterase present in the brain of the honey bee, *Apis mellifera* L., and in the nerve cords of the American cockroach, *Periplaneta americana* (L.). Only one substrate concentration was used. Tobias, Kollros, and Savit (1946) confirmed the observation so far as the cockroach nerve cord was concerned. The present authors (Babers and Pratt, 1950), also using only one substrate concentration, found, in contrast, that the cholinesterase in the head of the house fly, *Musca domestica* L., at similar substrate concentrations, split acetylcholine much faster than it did the methyl derivative. This work suggested that the enzymes of the cockroach, the honey bee, and the house fly capable of splitting acetylcholine esters were different. Metcalf and March (1949) at about the same time also observed a difference between bee and fly cholinesterases.

It is now generally accepted that a family of cholinesterases exists and that the members differ considerably in their properties. Serum cholinesterase, for instance, is different from that found in the erythrocytes. The specificity of these enzymes has been the subject of several polemics. Augustinsson (1948) has suggested that the enzymes be divided into two groups, as follows: (1) those enzymes whose activity is inhibited by excess substrate (this group would include the enzymes that have been described from the various nervous systems and erythrocytes; the maximum activity occurs when the substrate is about 3×10^{-3} M) and (2) the enzymes whose activity follows the Michaelis-Menten (1913) formulation and whose activities are maximum at infinite substrate concentration (the serum cholinesterases are included in this group).

Triacetin is split by the first group at a low rate at the concentration optimum for acetylcholine, but at high triacetin concentration the rate of splitting may be higher than that of acetylcholine. The erythrocyte esterase splits acetyl-β-methylcholine more slowly than acetylcholine at low substrate concentration but more rapidly when substrate concentrations are high. Thus, although Augustinsson (1948, 1949) has shown that both esters inhibit the enzyme in high concentration, the optimum substrate concentration is not the same for the two compounds. This author has emphasized the importance of studying the activity of cholinesterase at several substrate concentrations.

Straus and Goldstein (1943) and Goldstein (1944) have also emphasized the fallacies in the classical treatment of enzyme reactions where the reactions are considered to be of first order only. Along with other inhibitors, the authors studied the inhibiting effect of excess substrate.

The cholinesterase system in insects is of particular interest because of its in-

hibition by several of the more important insecticides (Chadwick and Hill, 1947; Roeder, 1948; Metcalf and March, 1949). For this reason it seemed desirable to investigate further the differences in the enzymes in several species of insects.

MATERIAL AND METHODS

House flies were reared in the laboratory on standard larval medium (Anonymous, 1949), and the adults were fed the gelatin-milk formula[1] in use at the laboratory of the Bureau of Entomology and Plant Quarantine, at Orlando, Florida.

TABLE 1

CHOLINESTERASE ACTIVITY OF WHOLE BREI VERSUS SUPERNATANT OF 30 PER CENT GLYCEROL EXTRACTS OF THE HEADS OF THE FLY AND HONEY BEE

Substrate 0.01 M	Carbon Dioxide Liberated per Minute (Cu. Mm.)			
	House Fly		Honey Bee	
	Supernatant Only	Whole Brei	Supernatant Only	Whole Brei
ACH.....	3.7	4.7	0.8	4.3
MeCH.....	1.2	1.3	0.6	3.5
TA.....	3.8	4.2	1.4	6.4

The heads of numerous adult insects were removed while the insects were under carbon dioxide anesthesia. The heads were then weighed, homogenized in a glass Potter-Elvehjem apparatus, and diluted to the desired volume with 30 per cent glycerol. The brei was then strained through cheesecloth to remove the few pieces of coarse chitinous material, a few drops of toluene were added, and the material was stored in the refrigerator. From freshly prepared extracts, it was determined that toluene did not inhibit the enzymatic activity. The concentrations of tissue used per milliliter for the different insects were as follows: house fly, 20 mg.; honey bee, 100 mg.; and cockroach, 100 mg.

Cholinesterase activity was determined by the usual Ammon (1933) technique, using standard Warburg apparatus. In the main compartment of the flask 0.5 ml. of brei was added to 2 ml. of modified Ringer's solution of such strength that, at final dilution, the suspension was 0.04 M magnesium chloride, 0.15 M sodium chloride, and 0.031 M sodium bicarbonate. One-half milliliter of substrate, prepared by dissolving the ester in distilled water, was placed in the side arm. The flasks were swept with a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide gas and placed in the bath at 37° C. After equilibration, the contents of the side arm were added to those in the main compartment, and, after 2 minutes, readings were made in the usual manner. To determine the reproducibility of the method, fifteen determinations were made of the activity of a house fly head preparation against acetylcholine bromide. The mean activity was 6.05 ± 0.159 cu. mm. carbon dioxide per minute.

For the sake of brevity throughout the discussion, acetylcholine bromide will be referred to as "ACH"; acetyl-β-methylcholine chloride as "MeCH"; and triacetin as "TA."

RESULTS

Solubility of the enzyme.—When the brei from the house fly or the honey bee was separated by centrifugation into a solid portion and clear supernatant, a difference in solubility of the enzymes was apparent. The results are shown in Table 1.

Thus, with the house fly, most of the

[1] 20 gm. of Bacto agar, 40 gm. of dehydrated banana powder, 300 gm. of sugar, 150 gm. of powdered whole milk, 50 gm. of gelatin, 1 ml. of 40 per cent formaldehyde, and 3 liters of water.

enzyme activity was in the supernatant. In the honey bee the reverse was true, since little activity remained in the supernatant after centrifugation of the whole brei.

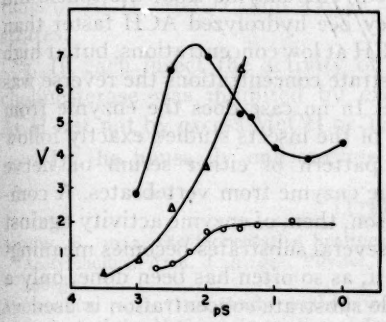

FIG. 1.—Activity-*pS* curves for the enzymic hydrolysis of ACH (●); MeCH (○); and TA (△); by the esterase in the heads of house flies.

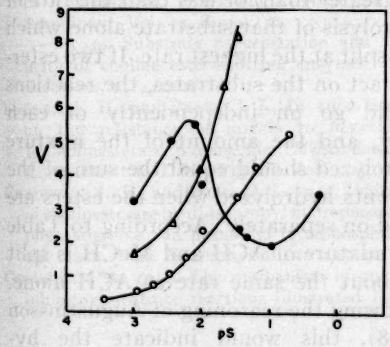

FIG. 2.—Activity-*pS* curves for the enzymic hydrolysis of ACH (●); MeCH (○); and TA (△); by the esterase in the heads of worker honey bees.

Activity curves.—Activity-*pS* curves for the enzymic decomposition of several substrates by the enzymes in the tissue of the heads of house flies are given in Figure 1, of honey bees in Figure 2, and of cockroaches in Figure 3. In each case the abscissa, *pS*, is the negative logarithm of the molar concentration of the substrate, and the ordinate, *V*, the average number of cubic millimeters of carbon dioxide liberated per minute.

Effect of tissue concentration and mixed substrates on rate of hydrolysis.—With ACH as the substrate, the effect of using various quantities of fly-head tissue is shown in Figure 4. It is obvious that the rate of decomposition is a function of the tissue concentration.

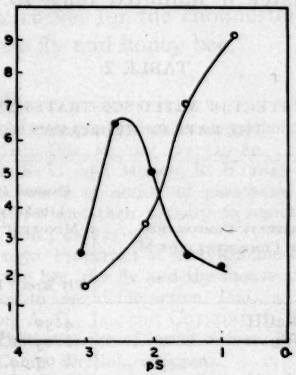

FIG. 3.—Activity-*pS* curves for the enzymic hydrolysis of ACH (●) and MeCH (○); by the esterase in the head of the cockroach.

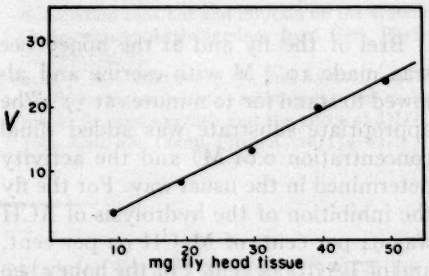

FIG. 4.—The effect of different concentrations of fly head tissue on the enzymic rate of decomposition of ACH.

In Table 2 the volume of carbon dioxide liberated by different mixed substrates is shown.

Inhibition of the enzyme.—The concentration of eserine required for 50 per cent inhibition (In_{50}) of the hydrolysis

of acetylcholine bromide was determined in the usual manner. For the esterase of the house-fly head In_{50} was 2.3×10^{-6} M, and for the honey bee, 3.2×10^{-6} M. In the course of these experiments it was noted that acetone solutions of parathion rapidly lost their power to inhibit the enzyme. A solution when freshly prepared inhibited 71.07 per cent, and 3 days later it inhibited only 20.78 per cent.

TABLE 2

EFFECT OF MITED SUBSTRATES ON THE RATE OF HYDROLYSIS

SUBSTRATE COMPOSITION (EACH COMPONENT 0.01 M)	CARBON DIOXIDE LIBERATED PER MINUTE (CU. MM.)	
	Fly Brei	Bee Brei
ACH+MeCH	4.50	4.05
ACH+TA	6.23	5.56
ACH	4.95	2.45
MeCH	1.50	1.50
TA	4.93	4.11
ACH+MeCH+10^{-6} eserine	0.15	.....
ACH+10^{-6} eserine	0.50	.....
MeCH+10^{-6} eserine	0	.....

Brei of the fly and of the honey bee was made 10^{-6} M with eserine and allowed to stand for 30 minutes at 37°. The appropriate substrate was added (final concentration 0.01 M) and the activity determined in the usual way. For the fly the inhibition of the hydrolysis of ACH was 94 per cent, of MeCH 92 per cent, and of TA 93 per cent. For the honey bee the respective percentages were 72, 80, and 72.

DISCUSSION

The activity-substrate concentration curve obtained when ACH is the substrate is bell-shaped, showing inhibition of enzymatic activity by excess substrate. With MeCH and TA, the curves are sigmoid, and the rate of reaction is generally proportional to the substrate concentration. In the case of the enzyme from the house fly, ACH was hydrolyzed faster than MeCH at all concentrations tested. The enzyme from the roach and honey bee hydrolyzed ACH faster than MeCH at low concentrations, but at high substrate concentrations the reverse was true. In no case does the enzyme from any of the insects studied exactly follow the pattern of either serum or nerve tissue enzyme from vertebrates. A comparison, then, of enzyme activity against the several substrates becomes meaningless if, as so often has been done, only a single substrate concentration is used.

If an enzyme preparation capable of catalyzing the hydrolysis of two substrates is allowed to act on a mixture of the two, the reaction rate might be equal to, greater than, or less than the rate of hydrolysis of that substrate alone which was split at the highest rate. If two esterases act on the substrates, the reactions should go on independently of each other, and the amount of the mixture hydrolyzed should equal the sum of the amounts hydrolyzed when the esters are acted on separately. According to Table 2, a mixture of ACH and MeCH is split at about the same rate as ACH alone. Following the reasoning of Augustinsson (1948), this would indicate the hydrolysis of both substrates by one enzyme. Similarly with the bee, the rate for the mixed substrate ACH plus MeCH is about equal to the sum of the rates of the separate substrates, indicating that two enzymes are acting independently. In the case of the mixture of ACH and *TA*, some inhibition apparently occurs, since neither with bee nor with fly brei does the amount of carbon dioxide liberated from the mixture equal that from the two components used alone.

SUMMARY

1. The enzymatic activities of the cholinesterase in the heads of the house fly, the honey bee, and the cockroach were compared, using various concentrations of ACH, MeCH, and TA as substrates.

2. In each insect the activity of the cholinesterase was inhibited by excess ACH but not by excess MeCH or TA.

3. In the house fly one enzyme apparently split both acetylcholine and the methyl derivative. In the honey bee two enzymes were apparently present.

4. The importance of using several substrate concentrations when attempting to classify cholinesterase activity is emphasized.

Since the submission of this manuscript for publication, Metcalf and March (1950) have obtained similar pS-activity curves for the cholinesterase of the house fly and honey bee.

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REACTION THRESHOLDS OF AN AQUATIC BEETLE, LACCOPHILUS MACULOSUS GERM., TO SALTS AND ALCOHOLS

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STUDY of the behavior and other phases of the physiology of amphibious organisms may eventually resolve a long-standing problem in chemoreception: the relation between the physical state of a chemical and its effectiveness in stimulating gustatory and olfactory receptors. This problem has been recognized since Matthes (1924), Strieck (1925), Von Frisch

(1924), and many other investigators argued effectively against the belief expressed by Nagel (1894) that olfactory receptors are stimulated only by gases and that aquatic animals must be restricted to a gustatory sense. A quantitative study of the effectiveness of the same chemical stimulant acting in both gaseous and liquid phases on both olfactory and gustatory receptors is lacking.

Certain aquatic beetles, being amphibious as adults and possessing chemoreceptors located on accessible mouth parts, appear to be ideally suited for use in such a study. Schaller (1926) reported that coumarin and synthetic musk, both effective stimuli in underwater conditioning experiments with *Dytiscus marginalis*, also stimulated as vapors when the beetles came out of water. The quantities of the stimulating substances were not controlled and the receptors involved questionable, in view of contradictory results of Ritter (1936) and Bauer (1938) with the same and related species.

Methods have already been devised to test the responses of insects to quantitatively measured gases (Dethier, 1947). The greatest difficulty which has prevented the further experimental use of aquatic insects has been the lack of a method for accurately controlling the quantity of the chemical stimulant in solution. Such a method must be employed not only in order to make all tests with solutions near threshold and avoid stimulation of the common chemical sense but also to permit comparison with other aquatic or terrestrial animals and thus discover any exceptional sensitivities among species having the dual air-water habitat. It is the purpose of this report to describe a technique and group of experiments making possible such comparisons, as a foundation for subsequent study of the larger problem initially stated. This experimental approach to the problem was suggested by Dr. V. G. Dethier, to whom the author is deeply indebted also for helpful criticisms during the progress of the work.

MATERIAL AMD METHODS

Laccophilus maculosus Germ. (identified through the courtesy of Dr. E. A. Chapin, of the United States National Museum) was chosen as the experimental animal because of its availability, size, and readiness to leave the water occasionally. Its inclusion in the family Dytiscidae, used by the German workers previously cited, is an advantage not only for comparing experimental results but also because of the wealth of anatomical and other information made available through Korschelt's (1923) monograph upon *D. marginalis*.

The beetles were netted during late summer along the shallow margins of fish-rearing ponds maintained by the Delaware State Game and Fish Commission. Until used in the experiments, the beetles were kept in aquaria provided with wooden floats, allowing them to dry themselves at intervals. One 40-gallon aquarium accommodated about one thousand beetles without overcrowding. All aquaria were covered with screens to prevent the escape of flying beetles. Meat scraps and fish foods were fed daily. Large initial stocks were necessary because an estimated 10 per cent of the beetles fell victim each month to cannibalism, which no amount of feeding eradicated.

Experiments were run in the morning on beetles maximally fed about an hour before use. Every effort was made to standardize the handling of the animals for all the tests. Once removed from the apparatus after being tested, the beetles were kept apart from untested stocks and were never used again in the experiments.

The testing apparatus was a modifica-

tion of one used by Jones (1948) in studying reactions of fish to toxic solutions. The following description refers to Figure 1. Water inlet (*A*) keeps 20-liter aspirators (*B*) filled. Constant water levels are maintained by overflow tubes (*C*). Water flows into the mixing funnel (*D*) at the rate of 500 ml/min, held constant by screw clamps (*1*); 1,000-ml. aspirators (*E*), filled to an experimentally determined level with concentrated test solution or with tap water (control), also deliver solution into the funnels at a known rate. When pinch clamps (*2*) are first opened, the rate of flow of the concentrate is higher than later, owing to a gradual loss of head, but the exact dilution during brief time intervals can be tested by sampling directly from the reaction tube (*I*), as will be described. The mixture passes through flush tubes (*F*), large air bubbles and some excess liquid are removed by tubes (*G*), which aid in keeping the head constant on both sides of the apparatus. Remaining air bubbles are removed in bubble traps (*H*), from which the solution flows directly into the reaction tube (*I*). This tube is 1 inch in diameter and 15 inches long. The solution enters the ends of the tube and is directed back against the rubber stoppers plugging the tube, following which the solution moves to the center of the tube, to be withdrawn by the exhausts (*J*). The exhaust flow from the center of the reaction tube maintains a sharp interface between solutions in the two halves of this tube. The transition zone between two solutions in opposite halves of the reaction tube is less than $\frac{1}{4}$ inch (measured parallel to the long axis of the reaction tube). This was shown by introducing concentrated dye solutions into one side of the tube. The flow of the exhaust from the reaction tube is adjusted to 500 ml/min by a screw clamp (*3*). By adjusting air exhausts (*K*), a narrow bubble of air can be kept at the top of the tube, from which the beetles can replenish their air supply. This bubble does not interfere with the maintenance of the interface between test and control solutions. Rotation of the air exhaust tubes makes possible the withdrawal of samples to check the solution concentration at different points along the surface of the stoppers in the ends of the reaction tube. HCl and NaOH concentrations were determined by titration of samples removed through *K*, and these concentrations were plotted as percentages of the original concentration introduced into the mixing funnel from one of the small aspirators. During the interval between 30 and 45 seconds, from the start of the flow of the concentrate into the mixing funnel, the concentration at the rubber stoppers in the reaction tube equals 50 per cent of the concentration introduced into the mixing funnel. With the flow from the 20-liter aspirators and from the reaction tube exhaust checked daily, the dilution error of the apparatus during the initial 30–45-second interval was within 3 per cent of the 50 per cent shown in Figure 2 and used in the calculations.

Before each day's experiments, tap water (pH about 7.8) was run through the apparatus for 1–2 hours, during which the temperature of the water fell to 14°–18° C. and remained within that range until the experiment was finished. Twenty-three beetles were used in the reaction tube at once. They were inserted by temporarily removing one of the end rubber stoppers. When water is run into the closed tube, the beetles typically swim to the ends and sit motionless on the stoppers. A light above the middle of the tube facilitates the counting of the beetles and usually keeps them from hanging onto the plastic screens placed over the ends of the exhaust tubes. Into the bal-

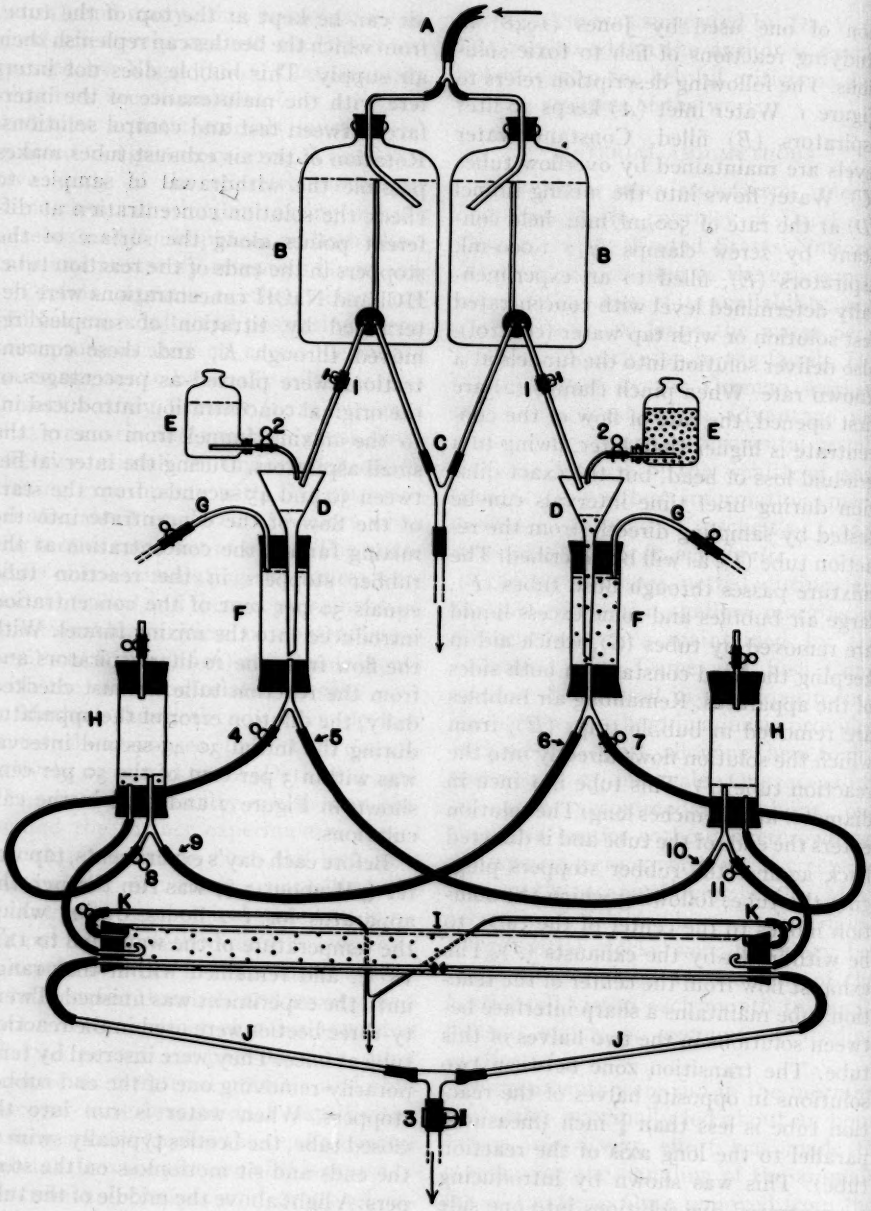

FIG. 1.—Design of apparatus. For explanation, see text

anced system of flowing tap water, entering the ends of the reaction tube, a solution (in tap water) of the compound to be tested is introduced from one 1,000-ml. aspirator. The solution is prepared so that its concentration in the small aspirator is exactly twice the concentration desired in the end of the reaction tube. The test solution is not buffered. Thus there is avoided a possible summation of chemical stimuli which would be an especially troublesome source of error, considering the low thresholds which were found for *Laccophilus*. Simultaneously with the introduction of the test solution, a tap-water control is introduced from the other 1,000-ml. aspirator. The position of the pinch clamps just below the flush tubes and the choice of the mixing funnels that the small aspirators supply determine which end of the reaction tube will receive each of the two solutions. The use of clamps in positions *5*, *6*, *9*, and *10*, rather than at *4*, *7*, *8*, and *11*, as shown in Figure 1 (but with small aspirators exactly the same) would deliver the test solution to the right end of the tube and the tap-water control to the left end.

Beetles react to the test solution by leaving their positions on the stoppers and swimming about in the tube. The largest number of beetles leaving the stopper during the initial 30–45-second interval was recorded as the number for which that concentration of the test solution was at or above the threshold. Doubtful responses were carefully and uniformly interpreted. At least 10 minutes elapsed between successive exposures to the test solutions.

It must be noted at the outset that the reaction of an entire organism, as occurs in this case, would not be expected to show a perfect correlation with the sensitivity of the chemoreceptors being stimulated. By employing this criterion for determining thresholds of chemoreceptors, the events between the receptors and a relatively complicated activity of the stimulated animals remain for future study. The data indicate, however, a good correlation between quantity of stimulating solution and proportion of beetles reacting (see "Results"). This correlation, together with relationships found between thresholds and the physical and chemical properties of the stimulating compounds, is evidence of the utility of the criterion of the threshold.

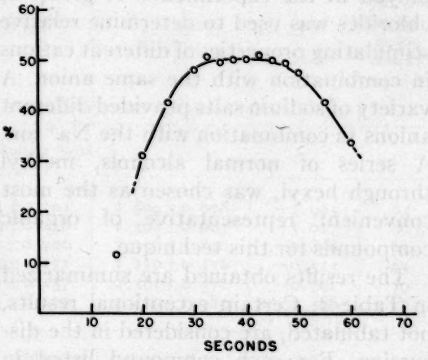

FIG. 2.—Time-dilution curve of the apparatus. For explanation, see text.

These same relationships also indicate that the experiments dealt primarily with the receptor cells rather than with some unknown link in the physiological processes intervening between the stimulation of the receptors and the reaction on which the measurements depended.

Plots from which the median reaction threshold concentrations were calculated were usually based upon tests of six doubling concentrations, the percentage reacting at each concentration representing the total number of beetles reacting in three different groups divided by the total number in those groups exposed to that particular concentration. Each percentage reaction is converted into probability units. A plot of these probits

against log concentration yields a linear regression (see "Results"). The position and slope of the regression line, the most probable value of the concentration required for 50 per cent reaction, and the variances of the line and of the median concentration are calculated by ordinary statistical methods. This procedure for analyzing the data is essentially that of Bliss (1938).

RESULTS

Three sets of compounds were employed in the experiments. A group of chlorides was used to determine relative stimulating properties of different cations in combination with the same anion. A variety of sodium salts provided different anions in combination with the Na^+ ion. A series of normal alcohols, methyl through hexyl, was chosen as the most convenient representative of organic compounds for this technique.

The results obtained are summarized in Table 1. Certain exceptional results, not tabulated, are considered in the discussion. For each compound listed in Table 1, the proportion of beetles reacting is directly related to the logarithm of the concentration. Data illustrating this relationship for one compound from each of the three groups used are presented graphically in Figure 3. Similar regression lines, illustrating this same relationship, can be drawn for any of the other compounds tested by using the data in the fourth, fifth, and sixth columns of Table 1 (see * note to Table 1).

DISCUSSION

The relationship between the percentage of the beetles reacting and the logarithm of the concentration of chemical stimulant is identical with that commonly noted among invertebrates. Dethier and Chadwick (1948) have demonstrated this phenomenon with their own data for the blowfly and by reanalyzing the data of other workers with various insects. They have also pointed out that the phenomenon is not peculiar to insects, since it is indicated also in the results of Krinner (1934) with the minnow *Phoxinus*. At present, information on the underlying reasons for this relationship is lacking.

The relative stimulating effectiveness of the anion series is, within the limits of the experimental error, similar to that found by Frings (1946) for the cockroach (*Periplanata americana*). This series is as follows: $OH^- \gg I^- > Br^- > SO_4^=$, CH_3COO^-, $Cl^- > PO_4^=$. Hopkins (1932) reported a similar series for the oyster (*Ostrea virginica*), with the latent period of tentacular response as the criterion for measuring the effectiveness of stimulation. This arrangement of anions has not yet been correlated with the physical properties of the ions.

The cation series, on the other hand, has been correlated with ionic mobility by Frings (1946). His series for the cockroach is: $H_3O^+ \ggg NH_4^+ > Rb^+ = Cs^+ \geq K^+ > Sr^{++} = Ca^{++} > Mg^{++} > Na^+ > Li^+$. The chlorides reported in Table 1 arrange themselves in the same series as that found by Frings. However, the thresholds for some of the chlorides used by Frings could not be determined for *Lactobacillus* with the methods described above. $CaCl_2$ and $MgCl_2$ consistently failed to evoke typical reactions. Even at concentrations above the ordinary physiological range, the beetles reacted only by crawling about on the stoppers and moving their antennae and mouth parts. In scattered instances they swam about in the tube, but the number doing so was insufficient to supply reliable data. The pH of these solutions was about 7.5, just as were the NaCl and KCl solutions. Frings found no significant change in threshold of $CaCl_2$ or NH_4Cl when the

pH was adjusted to that of his NaCl solution (pH 6.6); similar adjustment of the pH of $MgCl_2$ solutions raised, rather than lowered, the threshold. The atypical responses of *Laccophilus* described above for $CaCl_2$ and $MgCl_2$ were also observed with $CuCl_2$, $FeCl_2$, and $BaCl_2$. The latter three compounds were not among those tested on the oyster (Hopkins, 1932). The cation series based upon latent period of response of the oyster is as follows: $K^+ > NH_4^+ > Na^+ > Li^+$, similar to the cockroach and *Laccophilus*. $MgCl_2$ is listed among the salts least stimulating for the oyster. It would be interesting to know where $CaCl_2$ and the metallic

TABLE 1

REACTION OF *Laccophilus* TO SALTS AND NORMAL ALCOHOLS

COMPOUND	MOLAR CONC. EVOKING REACTION FROM 50 PER CENT OF BEETLES (THRESHOLD)	LOG THRESHOLD (± 2.575 S.E.)	$a \pm$ S.E.*	$b \pm$ S.E.*	$\bar{x}$*	NO. OF BEETLE-EXPOSURES†
			Sodium Salts			
NaOH	0.010	−1.986 ± 0.214	4.754 ± 0.162	2.804 ± 0.608	−1.995	82
NaI	0.098	−1.010 ± 0.162	4.984 ± 0.150	2.394 ± 0.605	−1.017	86
NaBr	0.14	−0.843 ± 0.257	5.036 ± 0.200	7.356 ± 1.890	−0.838	63
Na acetate	0.24	−0.615 ± 0.149	4.673 ± 0.175	3.220 ± 0.476	−0.513	73
Na_2SO_4	0.25	−0.608 ± 0.780	4.587 ± 0.157	2.261 ± 0.578	−0.791	79
Na phosphate	0.47	−0.323 ± 0.162	4.712 ± 0.174	4.470 ± 1.080	−0.388	76
			Chlorides			
HCl	0.0044	−2.357 ± 0.765	4.773 ± 0.125	1.409 ± 0.270	−2.520	132
NH_4Cl	0.046	−1.337 ± 0.145	5.029 ± 0.119	2.110 ± 0.285	−1.351	153
KCl	0.078	−1.106 ± 0.190	5.035 ± 0.108	1.461 ± 0.231	−1.082	170
NaCl	0.28	−0.558 ± 0.524	4.639 ± 0.131	1.430 ± 0.321	−0.810	115
LiCl	0.46	−0.340 ± 0.551	5.486 ± 0.162	1.587 ± 0.898	−0.265	97
			Alcohols			
Methanol	3.6	0.563 ± 0.466	4.523 ± 0.373	2.700 ± 0.501	0.387	156
Ethanol	4.3	0.639 ± 0.209	5.179 ± 0.106	2.361 ± 0.355	0.091	210
1-Propanol	3.2	0.496 ± 0.091	4.893 ± 0.109	3.144 ± 0.446	−0.530	165
1-Butanol	0.046	−1.334 ± 0.109	4.790 ± 0.310	2.444 ± 0.352	−1.420	219
1-Pentanol	0.0073	−2.133 ± 0.118	4.845 ± 0.103	2.311 ± 0.400	−2.201	173
1-Hexanol	0.0011	−2.941 ± 0.181	4.640 ± 0.096	2.288 ± 0.305	−2.898	225
			Miscellaneous			
$Be(NO_3)_2$	0.16	−0.810 ± 0.260	4.722 ± 0.206	2.256 ± 0.790	−0.933	50
$AgNO_3$	0.034	−1.474 ± 1.097	5.775 ± 0.215	1.173 ± 0.684	−0.813	47

* The fourth, fifth, and sixth columns of the table give the calculated values for a, b, and $\bar{x}$ in the equation $Y = a + b(X - \bar{x})$, which is the regression of per cent of beetles reacting (Y), expressed as probits, on log concentration (X). S.E. = standard error.

† 1 beetle-exposure = exposure of 1 beetle to any concentration of the test solution. Note that this figure does not indicate the number of different beetles used, which was 69 for all the compounds.

chlorides would fit into this series obtained with the oyster.

In attempting to account for these exceptional results observed with *Laccophilus*, the divalent structures or higher molecular weights of the test compounds were suspected of being somehow involved. That neither of these properties alone accounts for the atypical reaction is shown by the entirely normal reaction to

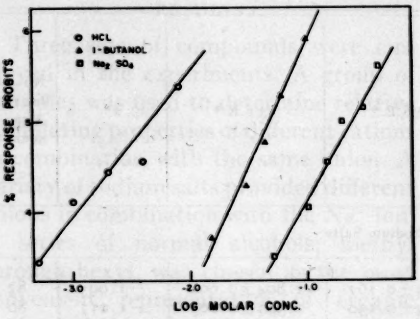

FIG. 3—Distribution of the percentage response probits as a function of concentration of the chemical stimulant.

$Be(NO_3)_2$, light and divalent, and of $AgNO_3$, monovalent but of molecular weight 170. To rely for explanation on specific physiological properties of these compounds or upon a combination of factors is not inconceivable but seems an alternative better deferred until their effect can be studied in other organisms related taxonomically or ecologically to *Laccophilus*. The same may be said of the possibility of ascribing the exceptional results to different effects in the central nervous system of the beetles or to any other factors which might modify the manner in which the beetles reacted.

Figure 4 shows the primary normal alcohol thresholds of *Laccophilus*. The curve describing these thresholds is strikingly similar to that reported for the blowfly (*Phormia regina* Meigen) by Dethier and Chadwick (1947, 1948). The inflection in the *Laccophilus* curve is at a point corresponding to 0.477 on the abscissa in Figure 4. This inflection at 1-propanol resembles the inflection at or beyond 1-butanol in the *Phormia* curve. A variety of physical properties, including thermodynamic characteristics, have been investigated in connection with the *Phormia* data, and a very high degree of correlation was found in many cases (Dethier and Chadwick, 1946). It is particularly interesting that the data for an organism in an aquatic environment are so similar to findings which show a high

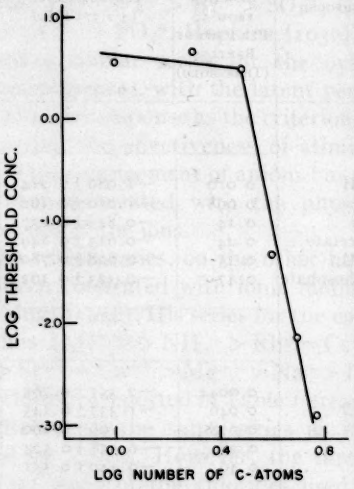

FIG. 4.—Reaction to alcohols as related to chain length of the stimulating compounds. (The slope of this *Laccophilus* curve for the lower alcohols is -0.277 ± 0.078; for the higher alcohols it is -13.330 ± 0.034.)

correlation with water-oil distribution coefficients, as is the case with *Phormia*. A lipid solubility-stimulation efficiency correlation would mean that compounds most likely to stimulate the receptors effectively would also be the ones least likely to occur in appreciable quantities within the organism's aqueous environment. All the alcohol thresholds of *Laccophilus* are much lower than those of the

blowfly. However, this is not entirely unexpected, since reactions of the blowfly were tested by the use of alcohol mixed with sugar. But the differences between thresholds of *Laccophilus* and the blowfly are greater for butanol and the longer-chain alcohols (see Table 2). Alcohol thresholds of the barnacle (*Balanus balanoides*), determined by Cole and Allison (1930) by using changes in cirral rhythm as the criterion for stimulation, are also much lower than those of the blowfly (see Table 2). The series tested on the barnacle did not include alcohols beyond 1-butanol, but a relatively greater difference between thresholds of the barnacle and of the blowfly for butanol (factor of 240, compared to 190 for shorter-chain alcohols) suggests that here, too, disproportionately increased sensitivity for compounds of higher partition coefficients may be established when additional data become available. It is possible that increased sensitivity to compounds less soluble in water is an adaptation to an aquatic existence which depends largely upon the use of chemoreceptors for awareness of the environment. The existence of such a dependence upon the chemoreceptors has been thoroughly established for aquatic beetles by Schaller (1926); and the extensive literature on chemical sensitivity of fish (Von Frisch, 1924; Strieck, 1925; Walker and Hasler, 1929) indicates that insects are not exceptional among aquatic animals in this respect.

The low thresholds of *Laccophilus* and their arrangement into series which are similar for other aquatic animals as well as the blowfly and cockroach indicate that this beetle is a promising species for future investigation. The comparisons seem to warrant the general conclusion that results obtained with *Laccophilus* may (with the exception of heavy divalent salts) be of general significance and not peculiar to this species. Whether the relatively great sensitivity to lipid soluble compounds tested in water is the same when these compounds are tested as gases remains to be shown. Along with these experiments on gaseous stimulation, the localization of the receptors responding to different categories of stimulants will have to be established, in order to restrict the chemoreceptors being compared to at least the same receptor group. Such experiments will shortly be carried out in this laboratory with *Laccophilus*.

TABLE 2

COMPARISON OF THE THRESHOLDS OF *Laccophilus*, *Phormia*, AND *Balanus* FOR NORMAL ALCOHOLS
(Molar Concentrations)

Alcohol	*Laccophilus* (*L*)	*Phormia* (*P*)*	*Balanus* (*B*)†	*P/L*	*P/B*
Methyl.	3.6	11.3	0.06	3.1	190
Ethyl...	4.3	3.2	0.017	0.7	190
Propyl..	3.2	1.3	0.0067	0.4	190
Butyl...	0.046	0.64	0.0027	13.9	240
Amyl...	0.0073	0.10	‡	13.7	.....
Hexyl..	0.0011	0.012	‡	10.9	.....

* Data for *Phormia* from Dethier and Chadwick (1948).
† Data for *Balanus* from Cole and Allison (1930).
‡ Data not available.

SUMMARY

1. A method for determining quantitative thresholds of aquatic beetles to aqueous solutions is described. The technique was used to establish reaction thresholds for *L. maculosus* Germ.

2. Cations in uniform anion combination stimulated in the following order of effectiveness: $H_3O^+ \gg NH_4^+ > K^+ > Na^+ > Li^+$. This is the order of ionic mobilities.

3. Anions in uniform cation combination stimulated in the following order of

effectiveness: $OH^- \gg I^- > Br^- > SO_4^=$, CH_3COO^-, $Cl^- > PO_4^=$. This order is as yet an empirical one.

4. Thresholds to normal alcohols decreased with increase in CH_2 groups. An inflection occurs in the curve relating logarithm of threshold concentration of alcohol to logarithm of chain length at 1-propanol. Stimulation is apparently directly related to lipoid solubility of the alcohols, as in the blowfly.

5. *Laccophilus* fails to give typical reactions to $CaCl_2$, $MgCl_2$, $CuCl_2$, $FeCl_2$, and $BaCl_2$ even at concentrations above the ordinary physiological range. The reason for this is not known, but it is not related to divalency or high molecular weight per se.

6. It is possible that, when stimulation depends upon lipoid solubility, the low concentrations likely to be present in aqueous solution can be detected by aquatic animals, owing to disproportionately lowered thresholds for this type of compound, when the aquatic forms are compared with nonaquatic relatives. Such an adaptation is indicated in *Laccophilus* as compared with the blowfly and, as data become available, may be revealed as a peculiarity of aquatic species in general.

7. The findings are discussed as a basis for subsequent experiments on the relation between the physical state of a chemical and its ability to stimulate gustatory and olfactory receptors.

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EFFECTS OF NUTRIENTS ON TETRAHYMENA GELEII W POPULATIONS[1]

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For some years investigators have successfully grown *Tetrahymena geleii* Furgason in bacteria-free culture. Until the papers of Furgason (1940) and Kidder and Dewey (1945) were published, however, a confusion of nomenclature made it difficult to evaluate the experimental studies on this ciliate. The behavior of all *T. geleii* populations, nevertheless, has been essentially the same, in that the ciliate always exhibits these phases of growth: a period of logarithmic growth, a maximum period, and a period of decline and death. Explanations of this phenomenon have followed one of two theories. Woodruff early suggested that decline and death were the result of an accumulation of toxic metabolic wastes in the medium. This assumption, current for some years, is no longer widely accepted. The alternative theory proposes that specific components of the nutrient mass might be critical to the synthesis of new protoplasm, that their reduction would stop synthesis, though not maintenance. The work of Lwoff and Roukhelman (1926) gives some support to this view, although, in the main, it upholds the opinion of Woodruff. In their nitrogen analyses of cultures of *Glaucoma piriformis* (now *T. geleii*, according to Furgason, 1940), Lwoff and Roukhelman found that, while total nitrogen diminished only slightly, the peptone fraction decreased steadily; but they did not have culture media which would permit the accumulation of conclusive data.

Recently Kidder and Dewey (1949) developed a medium the components of which are chemically identified. This would have been preferable to proteose peptone as a basic medium in the present work, but peptone was chosen for two reasons: first, the exact nature of protogen had not been identified, and, as a result, Kidder and Dewey's complete list of constituents had not been published when this study was begun; and, second, proteose peptone (1.0 per cent) had been found suitable for culturing *T. geleii* (Baker and Huddleston, 1947), and it appeared to be a medium which might show specific effects of added nutrients. This study was planned to investigate qualitatively the effects of the individual components of the Kidder-Dewey medium on population growth of *T. geleii* W.

MATERIAL AND METHODS

In this investigation the organism used was *T. geleii* strain W (terminology of Kidder and Dewey, 1945), originally isolated from Mill Pond, Woods Hole, Massachusetts, in 1940, and established in pure culture by Dr. C. L. Claff at Brown University in the same year. Our stock culture of strain W originated from one which Dr. Austin Phelps, of the University of Texas, received from Dr. G. W.

[1] An investigation carried out in the Department of Biology at the Catholic University of America, Washington, D.C., in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The author wishes to acknowledge the kindness of Dr. E. G. S. Baker, who directed the study. She wishes to thank also Mr. W. F. Simpson and Mrs. B. H. McGovern, who, together with Dr. Baker, gave valuable advice and assistance.

Kidder and sent to Dr. E. G. S. Baker in 1947.

Throughout the study, stock cultures were maintained on Bacto-Proteose Peptone, Difco (hereafter designated "P.P."), 1.0 per cent, or a lower concentration, corresponding to that of a given experimental series. The P.P. was dissolved either in dilute Osterhout's solution, 0.012 per cent total salt (Johnson, 1933), or in the salt solution of the Kidder-Dewey complete medium (1949), in which latter case it is specifically stated in Tables 3 and 4. In the preparation of all culture media, double distilled water and Pyrex glassware, cleansed with Na_3PO_4 and thoroughly rinsed, were used. All P.P. was autoclaved at 15 pounds pressure for 20 minutes in Erlenmeyer flasks (50 ml. per flask), in which the *Tetrahymena* were subsequently grown. Experimental flasks were inoculated with *T. geleii* in the logarithmic phase of growth, as determined by previous counting. The volume of inoculum was that calculated to give an initial count of 1,000 organisms per milliliter, but exact numbers were determined by counting samples taken immediately after inoculation. Experimental cultures were incubated at 25° ± 3° C., but stock cultures were sometimes kept at 15° ± 2° C., to prolong life—a procedure considered permissible on the basis of previous observations (Baker and Schleicher, 1948). Bacteriological technique was used in all experiments. To determine the pH, a Beckman pH meter was employed, but, because the variability of the pH of 1.0 per cent P.P. was so slight as to be negligible in our work—even after the addition of a single nutrient—determinations were made only when deemed necessary.

For all experiments, both amino acids and vitamins were prepared fresh each time; care was taken to avoid exposing these nutrients to light (Kidder and Dewey, 1949). Although an attempt was made to approximate the amounts in the Kidder-Dewey complete medium, the approximation was not precise in all cases. In the experiments with amino acids the difference obviously was intentional; in others it was almost impossible to achieve the precise concentration per 1.0 ml., a limit necessary for keeping the total volume of P.P. plus nutrient at 50 ml. + 1.0 ml. The single amino acids were added to the P.P. before it was autoclaved; all other nutrients were sterilized with a sintered glass filter and added aseptically to the basic peptone medium.

For each of the single-nutrient series, A_1–A_{21} inclusive, three cultures were established: (1) a control (*Tetrahymena* in unsupplemented P.P. of the basic concentration); (2) a second flask of P.P. to which was added the specific nutrient at the time of protozoan inoculation; and (3) a third flask containing P.P. to which the nutrient was added at the estimated end of the logarithmic growth of *Tetrahymena*. These cultures will be referred to as Nos. 1, 2, and 3, respectively.

For the series of combinations of nutrients, Series B_1–B_5 inclusive, three cultures each were established in the same manner as for the single-nutrient series. In Series B_6 and B_7, four cultures were set up, the media of which are described in detail in connection with the observations on these series.

Initial samples were taken for counting at the time of inoculation, and subsequent samples at approximately every 12 hours for 2 days and at every 24 hours thereafter, until the beginning of population decline or longer. Population samples were counted in a Sedgwick-Rafter cell by using Johnson and Baker's application (1943) of the method originally developed by Hall, Johnson, and Loefer

(1935). To graph the population growth, logarithms of the numbers of ciliates per milliliter were plotted on the vertical axis against time on the horizontal axis (see Figs. 1–6).

OBSERVATIONS

I. SINGLE NUTRIENTS IN 1.0 PER CENT PROTEOSE PEPTONE, SERIES A_1–A_{21} INCLUSIVE

AMINO ACIDS

A_1.....l(+)arginine*
A_2.....l(+)histidine*
A_3.....dl-isoleucine*
A_4.....l(−)leucine*
A_5.....dl-methionine*
A_6.....l(+)lysine*
A_7.....dl-phenylalanine*
A_8.....l(−)tryptophane*
A_9.....dl-valine*
A_{10}.....dl-threonine*
A_{11}.....dl-serine

* Kidder-Dewey "essential amino acid."

GROWTH SUBSTANCES

A_{12}.....biotin
A_{13}.....pyridoxine HCl
A_{14}.....Ca pantothenate
A_{15}.....nicotinic acid
A_{16}.....riboflavine
A_{17}.....choline HCl
A_{18}.....pteroylglutamic acid
A_{19}.....nucleic acid
A_{20}.....thiamine in 1.0 per cent P.P.
A_{21}.....thiamine in 0.1 per cent P.P.

As can be seen in Tables 1 and 2, each of the proteose peptone cultures to which were added the single amino acids or growth substances exhibited a population increase or decrease to some degree. But only those cultures the deviation[2] of which is statistically significant will be discussed at any length in this paper. In several instances results which appear to be statistically significant are ignored because of slight contamination in the cultures, e.g., No. 3 of Series A_8, A_9, and A_{18} (cf. Tables 1 and 2). A comparison of the average maximal populations of the experimental cultures with the same populations of the controls gives the following data.

In the *single amino acid series*, No. 2 of Series A_3 (1.0 per cent P.P. + isoleucine) showed a population increase of 74,000 ciliates per milliliter. In No. 2 of Series A_{10} (1.0 per cent P.P. + threonine), the population was 26,000 higher per milliliter. On the other hand, in Series A_7 (1.0 per cent P.P. + phenylalanine), populations in Nos. 2 and 3 were 32,000 and 50,000 ciliates per milliliter lower, respectively. In Series A_9 (1.0 per cent P.P. + valine), there was also a decrease in No. 2 of 37,000 ciliates per milliliter.

In the *single growth substance series*, both experimental cultures in Series A_{16} (1.0 per cent P.P. + riboflavine) showed populations markedly higher than the control: growth in No. 2 was 34,000 ciliates per milliliter higher; in No. 3 there were 18,000 ciliates more per milliliter. The opposite phenomenon was observed in Series A_{18} (1.0 per cent P.P. + pteroylglutamic acid), flask No. 2 of this series having a decrease of 60,000 ciliates per milliliter. Similarly in Series A_{19} (1.0 per cent P.P. + nucleic acid), the population of No. 3 was 26,000 per milliliter lower than the control. It should be noted also that, although the population increases of both thiamine Series A_{20} and A_{21} are not statistically significant, the maximum stationary phase was prolonged in three out of four experimental cultures (cf. Table 2). It should be noted, too, that in Series A_{21} (0.1 per cent P.P. + thiamine) the phase of decline was more gradual in Nos. 2 and 3 than in No. 1 (cf. Fig. 3). Compared with Series A_{20} (1.0

[2] This means deviation of the average maximal population of an experimental culture from that of the control in the same series. To the average maximal populations expressed in numbers, the *t*-Test was applied. On the results of this test, the deviations were judged highly significant, significant, possibly significant, or not significant in terms of Davies' "significance levels" (cf. n. †, Table 1).

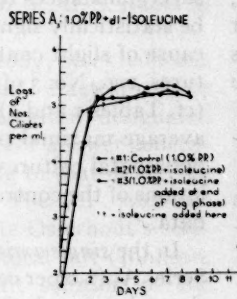
SERIES A: 1.0% PP + dl-ISOLEUCINE
Logs. of Nos. Ciliates per ml.
1 = #1: Control (1.0% PP)
2 = #2 (1.0% PP + isoleucine)
3 = #3 (1.0% PP + isoleucine added at end of log phase)
* = isoleucine added here
DAYS

FIG. 1

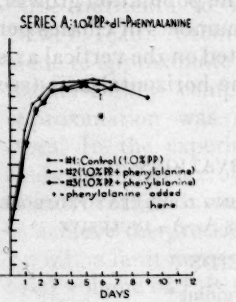
SERIES A: 1.0% PP + dl-PHENYLALANINE
1 = #1: Control (1.0% PP)
2 = #2 (1.0% PP + phenylalanine)
3 = #3 (1.0% PP + phenylalanine)
* = phenylalanine added here
DAYS

FIG. 2

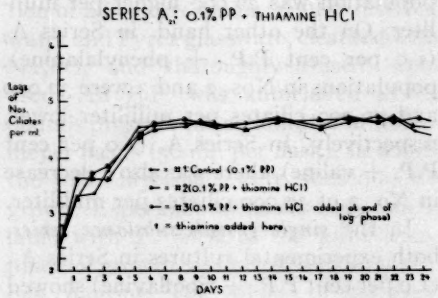
SERIES A: 0.1% PP + THIAMINE HCl
Logs. of Nos. Ciliates per ml.
1 = #1 Control (0.1% PP)
2 = #2 (0.1% PP + thiamine HCl)
3 = #3 (0.1% PP + thiamine HCl added at end of log phase)
* = thiamine added here.
DAYS

FIG. 3

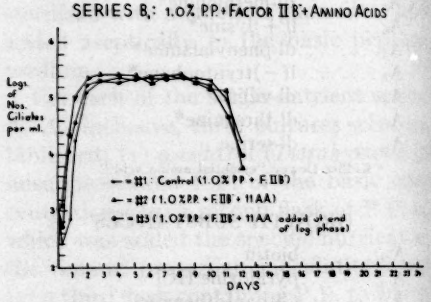
SERIES B: 1.0% PP + FACTOR II B' + AMINO ACIDS
Logs. of Nos. Ciliates per ml.
1 = #1: Control (1.0% PP + F. II B')
2 = #2 (1.0% PP + F. II B' + 11 AA)
3 = #3 (1.0% PP + F. II B' + 11 AA added at end of log phase)
DAYS

FIG. 4

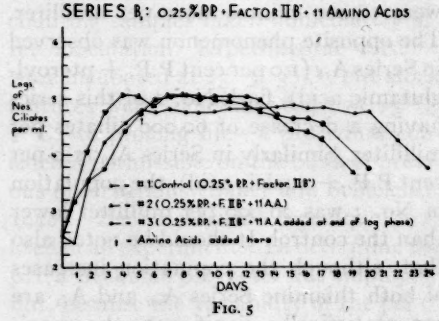
SERIES B: 0.25% PP + FACTOR II B' + 11 AMINO ACIDS
Logs. of Nos. Ciliates per ml.
1 = #1: Control (0.25% PP + Factor II B')
2 = #2 (0.25% PP + F. II B' + 11 A.A.)
3 = #3 (0.25% PP + F. II B' + 11 A.A. added at end of log phase)
* = Amino Acids added here
DAYS

FIG. 5

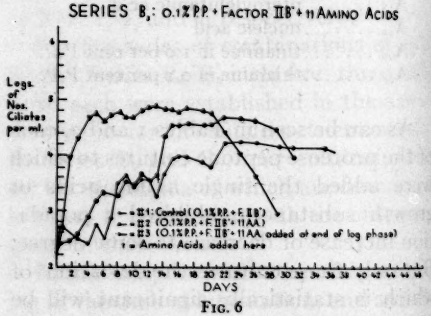
SERIES B: 0.1% PP + FACTOR II B' + 11 AMINO ACIDS
Logs. of Nos. Ciliates per ml.
1 = #1: Control (0.1% PP + F. II B')
2 = #2 (0.1% PP + F. II B' + 11 AA)
3 = #3 (0.1% PP + F. II B' + 11 AA added at end of log phase)
* = Amino Acids added here
DAYS

FIG. 6

FIGS. 1–6.—These figures are a graphic representation of the population growth of bacteria-free cultures of *T. geleii* W. The abscissae represent time, expressed in days. The ordinates represent the logarithms of numbers of ciliates per milliliter of medium. Flask No. 1 = control; Nos. 2 and 3 = experimental cultures. Data were taken from Tables 1–4, inclusive.

per cent P.P. + thiamine), maximal growth in Series A_{21} was approximately 80 per cent lower, and the life-span of the latter was nearly 337 hours longer than that of the former. (The significance of this will be emphasized later.)

2. COMBINATIONS OF AMINO ACIDS AND/OR GROWTH SUBSTANCES IN DIFFERENT CONCENTRATIONS OF PROTEOSE PEPTONE, SERIES B_1–B_7 INCLUSIVE

The combinations of amino acids and/or growth substances proved, in the

TABLE 1

PROTEOSE PEPTONE (1.0 PER CENT) PLUS SINGLE AMINO ACIDS

Series	Nos. of Cultures and Components of Media	Life-Span* (Hr.)	Log. Phase (Hr.)	Max. Phase (Hr.)	Avg. Max. Pop.	Results of *t*-Test†
A_1‡	1, 1.0% P.P. (control)	181	32	109	220,357	
	2, 1.0% P.P.+l(+)arginine	181	36	94	233,504	Not sig.
	3, 1.0% P.P.+l(+)arginine§	181	36	54	222,500	Not sig.
A_2	1, 1.0% P.P.	168	38	47	284,040	
	2, 1.0% P.P.+l(+)histidine	168	38	48	293,666	Not sig.
	3, 1.0% P.P.+l(+)histidine	168	38	47	256,120	Not sig.
A_3	1, 1.0% P.P.	179	36	83	142,092	
	2, 1.0% P.P.+dl-isoleucine	205	48	96	216,566	Hi. sig.
	3, 1.0% P.P.+dl-isoleucine	205	48	142	151,430	Not sig
A_4	1, 1.0% P.P.	162	36	85	187,856	
	2, 1.0% P.P.+l(−)leucine	162	36	65	208,955	Not sig.
	3, 1.0% P.P.+l(−)leucine	162	36	65	231,530	Not sig.
A_5	1, 1.0% P.P.	139	26	70	230,442	
	2, 1.0% P.P.+dl-methionine	169	26	103	194,586	Not sig.
	3, 1.0% P.P.+dl-methionine	169	26	73	213,370	Not sig.
A_6	1, 1.0% P.P.	184	39	89	272,823	
	2, 1.0% P.P.+l(+)lysine	160	39	72	243,562	Not sig.
	3, 1.0% P.P.+l(+)lysine	160	39	65	285,420	Not sig.
A_7	1, 1.0% P.P.	159	42	89	296,644	
	2, 1.0% P.P.+dl-phenylalanine	159	42	71	263,744	Psb. sig.
	3, 1.0% P.P.+dl-phenylalanine	218	42	71	245,888	Sig.
A_8	1, 1.0% P.P.	192	42	96	293,412	
	2, 1.0% P.P.+l(−)tryptophane	168	42	48	265,637	Not sig.
	3, 1.0% P.P.+l(−)tryptophane	192	42	96	267,352	Psb. sig.
A_9	1, 1.0% P.P.	192	42	96	293,412	
	2, 1.0% P.P.+dl-valine	168	40	72	255,847	Sig.
	3, 1.0% P.P.+dl-valine	216	40	119	266,352	Sig.
A_{10}	1, 1.0% P.P.	151	33	72	255,073	
	2, 1.0% P.P.+dl-threonine	151	33	72	281,522	Psb. sig.
	3, 1.0% P.P.+dl-threonine	151	33	72	239,960	Not sig.
A_{11}	1, 1.0% P.P.	164	48	50	224,290	
	2, 1.0% P.P.+dl-serine	164	48	50	231,410	Not sig.
	3, 1.0% P.P.+dl-serine	164	48	50	231,230	Not sig.

* Life-span = time in hours from initial growth to beginning of decline.

† Evaluated in terms of "significance levels" (Davies, 1947), namely: P less than 1 per cent = Highly significant; P between 5 and 1 per cent = Significant; P between 10 and 5 per cent = Possibly significant; P more than 10 per cent = Not significant.

‡ Amino acids in Series A_1 to A_{11} inclusive = Kidder-Dewey "essential."

§ To all No. 3 cultures of Table 1, amino acid added at estimated end of logarithmic growth of *T. geleii*.

main, to be stimulatory for *T. geleii* (cf. Tables 3 and 4). In all the experimental cultures of Series B_1, B_2, B_3, and B_4, as well as in flask No. 2 of Series B_6 and flask No. 4 of B_7, the rise in average maximal populations was statistically significant. Only flasks Nos. 4 of Series B_6 and 2 of Series B_7 showed any significant lowering of maximal populations.

In Series B_1 of this group of experiments, 1.0 per cent P.P. was dissolved in a solution of the Kidder-Dewey salts ($MgSO_4$, K_2HPO_4, $CaCl_2$, $MnCl_2$, $ZnCl_2$), to which the mixture of eleven amino acids—ten essential plus serine—and Factor IIB″ (Cu and Fe salts) were added. The latter is a component of Factor II—a growth factor obtained from liver and now known to consist of two fractions: Factor IIA, or protogen, which was concentrated by Stokstad *et al.* (1949), and Factor IIB, composed of

TABLE 2

PROTEOSE PEPTONE PLUS SINGLE GROWTH SUBSTANCES

Series	Nos. of Cultures and Components of Media	pH of Initial Media	Life-Span* (Hr.)	Log. Phase (Hr.)	Max. Phase (Hr.)	Avg. Max. Pop.	Results of *t*-Test†
A_{12}	1, 1.0% proteose peptone	6.75	166	42	68	250,680	
	2, 1.0% P.P.+biotin	.....	166	42	93	223,150	Hi. sig.
	3, 1.0% P.P.+biotin‡	.....	166	42	68	247,925	Not sig.
A_{13}	1, 1.0% P.P.	6.75	169	24	51	286,217	
	2, 1.0% P.P.+pyridoxine	.....	169	24	51	285,527	Not sig.
	3, 1.0% P.P.+pyridoxine	.....	193	24	76	263,960	Not sig.
A_{14}	1, 1.0% P.P.	6.75	182	39	72	291,317	
	2, 1.0% P.P +Ca pantothenate	.....	182	39	70	294,410	Not sig
	3, 1.0% P.P.+Ca pantothenate	.....	182	39	70	293,858	Not sig.
A_{15}	1, 1.0% P.P.	6.75	176	39	79	301,025	
	2, 1.0% P.P.+nicotinic acid	.....	152	39	79	284,498	Not sig.
	3, 1.0% P.P.+nicotinic acid	.....	176	39	80	282,800	Not sig.
A_{16}	1, 1.0% P.P.	6.97	168	42	96	225,688	
	2, 1.0% P.P.+riboflavine	.....	168	42	96	260,318	Sig.
	3, 1.0% P.P.+riboflavine	.....	168	42	96	243,902	Psb. sig.
A_{17}	1, 1.0% P.P.	6.78	162	39	74	271,370	
	2, 1.0% P.P.+choline HCl	.....	207	39	74	277,433	Not sig.
	3, 1.0% P.P.+choline HCl	.....	207	39	74	287,992	Not sig.
A_{18}	1, 1.0% P.P.	6.78	187	39	74	318,855	
	2, 1.0% P.P.+pteroylglutamic acid	6.99	216	39	149	258,490	Sig.
	3, 1.0% P.P.+pteroylglutamic acid	6.99	187	39	74	301,065	Psb. sig.
A_{19}	1, 1.0% P.P.	6.95	201	39	96	270,200	
	2, 1.0% P.P.+nucleic acid (yeast)	.....	201	33	96	257,078	Not sig.
	3, 1.0% P.P.+nucleic acid (yeast)	.....	201	48	120	243,617	Psb. sig.
A_{20}	1, 1.0% P.P.	6.89	197	46	95	266,718	
	2, 1.0% P.P.+thiamine HCl	6.88	246	46	144	290,570	Not sig.
	3, 1.0% P.P.+thiamine HCl	6.88	246	46	192	293,218	Not sig.
A_{21}	1, 0.1% P.P.	6.88	583	127	331	44,886	
	2, 0.1% P.P.+thiamine HCl	6.57	583	149	185	45,856	Not sig.
	3, 0.1% P.P.+thiamine HCl	6.57	655	120	434	45,726	Not sig.

* Life-span = time in hours from initial growth to beginning of decline.

† Cf. n. †, Table 1.

‡ In all No. 3 cultures of Table 2, growth substance added at estimated end of logarithmic growth of *T. geleii*.

fractions IIB′ and IIB″. Factor IIB′ is replaceable by high levels of pyridoxine, while Factor IIB″ may be replaced by Cu and Fe salts in relatively high concentrations (Kidder and Dewey, 1949). In this series the average maximal population was 11 per cent higher in No. 2 and 5 per cent higher in No. 3 than in the control. The maximum stationary phase in all three cultures of Series B_1 was 15–20 per cent longer than the same phase in the majority of cultures in Series A_1–A_{19}. In an attempt to determine the reason for this prolonged maximum phase, it was decided to use lower concentrations of P.P. To the peptone in each series, Factor IIB″ and the mixture of amino acids were added as in Series B_1.

In Series B_2 (0.25 per cent P.P. + amino acid mixture), exponential growth in No. 2 was higher than in both the control and No. 3. The amino acids had not yet been added to No. 3. The maximal population increase in Nos. 2 and 3 was 40 per cent above the same phase of

TABLE 3

PROTEOSE PEPTONE PLUS COMBINATIONS OF AMINO ACIDS AND/OR GROWTH SUBSTANCES

Series	Nos. of Cultures and Components of Media	pH of Media	Life-Span* (Hr.)	Log. Phase (Hr.)	Max. Phase (Hr.)	Avg. Max. Pop.	Results of *t*-Test†
B_1‡	1, 1.0% P.P.+Factor IIB″ (Cu and Fe salts)	6.79	198	51	120	238,487	
	2, 1.0% P.P.+{Factor IIB″ mixture 11 amino acids	6.63	219	39	128	270,228	Sig.
	3, 1.0% P.P.+{Factor IIB″ mixture 11 amino acids§	6.63	219	45	128	225,600	Not sig.
B_2‡	1, 0.25% P.P.+Factor IIB″	6.22	462	90	287	64,689	
	2, 0.25% P.P.+{Factor IIB″ mixture 11 amino acids	5.90	242	69	120	118,788	Hi. sig.
	3, 0.25% P.P.+{Factor IIB″ mixture 11 amino acids§	5.90	314	99	144	109,426	Hi. sig.
B_3‡	1, 0.1% P.P.+Factor IIB″	5.98	698	365	98	16,866	
	2, 0.1% P.P.+{Factor IIB″ mixture 11 amino acids	5.37	486	123	194	67,753	Hi. sig.
	3, 0.1% P.P.+{Factor IIB″ mixture 11 amino acids§	5.37	698	410	98	46,700	Hi. sig.
B_4	1, 1.0% P.P.+Factor IIB′ and IIB″	6.70	188	27	96	222,442	
	2, 1.0% P.P.+{Factor IIA (protogen) Factor IIB′ and IIB″	.....	166	27	72	252,642	Sig.
	3, 1.0% P.P.+{Factor IIA (protogen)§ Factor IIB′ and IIB″	.....	188	27	96	243,235	Sig.
B_5	1, 0.25% P.P.+Factor IIB′ and IIB″	6.68	239	26	175	121,971	
	2, 0.25% P.P.+{Factor IIA (protogen) Factor IIB′ and IIB″	.....	283	26	165	132,266	Not sig.
	3, 0.25% P.P.+{Factor IIA (protogen)§ Factor IIB′ and IIB″	.....	283	26	209	121,824	Not sig.

* Life-span = time in hours from initial growth to beginning of decline.
† Cf. n. †, Table 1.
‡ Solvent for P.P. in this series = solution of Kidder-Dewey salts.
§ Added at estimated end of logarithmic growth of *T. geleii*.

growth in the control. In all three cultures of Series B_2, there occurred (as in Series A_{21}, 0.1 per cent P.P. + thiamine) a coincidental lower maximal population, a prolonged maximum stationary phase, and a longer life-span (cf. Figs. 4 and 5). Actual cell measurement under the conditions of this study was not possible, but it was apparent during the counting process that the ciliates grown in 0.25 per cent P.P., in this and in subsequent experiments, were smaller than those grown in 1.0 per cent P.P. This was particularly noticeable in the later stages of a given 0.25 per cent culture.

This phenomenon—coincident lower population and longer life-span—was even more striking in Series B_3 (0.1 per cent P.P. + Factor IIB″ + the mixture of eleven amino acids). In No. 2 of Series B_1, 1.0 per cent P.P. cultures, the average maximal population was approximately 270,228 ciliates per milliliter, and the life-span was 219 hours; in No. 2 of Series B_2, 0.25 per cent P.P. cultures, the average maximal population was 118,788 per milliliter, the life-span 242 hours; in No. 2 of Series B_3, 0.1 per cent P.P. cultures, the average maximal population was 67,753 per milliliter, and the life-span was 486

TABLE 4

PROTEOSE PEPTONE PLUS COMBINATIONS OF AMINO ACIDS AND/OR GROWTH SUBSTANCES

Series	Nos. of Cultures and Components of Media	pH of Media	Life-Span* (Hr.)	Log. Phase (Hr.)	Max. Phase (Hr.)	Avg. Max. Pop.	Results of t-Test†
B_6	1, 0.25% P.P. in Osterhout's sol.	6.78	265	48	144	115,138	
	2, 0.25% P.P. in Osterhout's sol. + Mixture of 11 amino acids	6.49	217	48	71	130,278	Sig.
	3, 0.25% P.P. in Kidder-Dewey salt sol. + Factor IIB′ and IIB″, mixture of 11 amino acids	6.69	217	54	95	114,650	Not sig.
	4, 0.25% P.P. in Kidder-Dewey salt sol. + Factor IIA, Factor IIB′ and IIB″, mixture of 11 amino acids	6.69	217	56	71	101,204	Sig.
B_7	1, 0.25% P.P. in Kidder-Dewey salt sol. + Factor IIB″	6.85	387	94	223	96,239	
	2, 0.25% P.P. in Kidder-Dewey salt sol. + Group X A.A.: dl-serine, l(+)arginine, dl-valine	6.84	388	94	200	88,473	Sig.
	3, 0.25% P.P. in Kidder-Dewey salt sol. + Group Y A.A: dl-serine, l(+)histidine HCl, l(+)lysine HCl, dl-methionine, l(−)tryptophane	6.66	243	94	104	90,612	Not sig.
	4, 0.25% P.P. in Kidder-Dewey salt sol. + Group Z A.A: dl-serine, dl-isoleucine, l(−)leucine, dl-phenylalanine, dl-threonine	6.87	291	89	152	113,383	Hi. sig.

* Life-span = time in hours from initial growth to beginning of decline.

† Cf. n. †, Table 1.

hours. The fact that the beginning of decline began approximately 315 hours later rendered this correlation of lower population and longer life-span more conspicuous in Series B_3. This series demonstrated also that the amino acid mixture had a marked effect on the growth of *T. geleii* (cf. Fig. 6). In this series logarithmic growth was notably higher in No. 2, in which the amino acids were present at the outset, than in Nos. 1 and 3; and, although logarithmic growth in No. 3 ran close to that of the control, there was a second exponential growth period in No. 3 after the amino acids had been added. The results of Series B_3 cannot be attributed to the effect of suboptimal pH: the experimental cultures—the pH of which was 5.37, a value below the optimal range of 5.6–8.0 (Kidder, 1941)—showed greater population growth than the control, the pH of which was 5.98. Cell size, as well as populations, showed a marked decrease in 0.1 per cent P.P.; for, even without measurement, it was apparent that the Protozoa were smaller in Series B_3 than in Series B_2 or B_1.

Since lower concentrations of proteose peptone seemed to demonstrate the behavior of *T. geleii* more effectively, 0.25 per cent P.P. was used along with 1.0 per cent P.P. as basic media for further study of Factor II and the amino acids. In Series B_4 (1.0 per cent P.P. + Factor II complete), maximal growth in Nos. 2 and 3 exceeded that of the control by 11 per cent and 8 per cent, respectively. In Series B_5 (0.25 per cent P.P. + Factor II complete) Nos. 1 and 3, before the addition of protogen[3] (Factor IIA), exhibited the steepest logarithmic growth curve of any experimental series. In all three flasks the maximum stationary phase was exceptionally long: 175 hours in the control; 165 and 209 hours in Nos. 2 and 3, respectively.

Because previous experiments indicated that *T. geleii* population growth was influenced by Factor II and the amino acid mixture, added separately or in combination to P.P., Series B_6 and B_7 were planned to demonstrate further effects of these nutrients.

In Series B_6 four cultures were prepared with 0.25 per cent P.P. as the basic medium: No. 1 was set up as a control with unsupplemented P.P. dissolved in Osterhout's solution; in No. 2 only the amino acid mixture in half the amounts used by Kidder and Dewey (1949) was added to the peptone dissolved in Osterhout's solution; to the P.P. in No. 3, besides the amino acid mixture designated above for No. 2, were added Factor IIB′ (pyridoxine) and Factor IIB″ (Cu and Fe salts); the P.P. in No. 4 was supplemented with Factor IIA (protogen) and Factor IIB complete (pyridoxine and the Cu and Fe salts) and the same kinds and proportions of amino acids as in Nos. 2 and 3 above. As solvent for the P.P. in No. 3 and 4, a solution of the Kidder-Dewey salts was used as in Series B_1. Data for this series indicate that in flask No. 2 (containing the amino acid mixture in the absence of Factor II), maximal growth was significantly higher (15,140 per ml.) than in No. 1. Maximal growth in No. 4 was, by contrast, 13,934 ciliates per milliliter less. In No. 1, logarithmic growth was more rapid, the maximum stationary phase was longer, and the phase of decline was more gradual than in any of the other three cultures.

In Series B_7, the P.P. was dissolved in the Kidder-Dewey salt solution. To this

[3] The protogen was dissolved in 20 per cent ethyl alcohol, to give a maximum volume of 1.0 ml. to be added per 50 ml. P.P. The effect of the alcohol seemed negligible here; that in small amounts it has no effect on growth of Protozoa has been reported also by others. The protogen was obtained through the courtesy of Dr. E. L. R. Stokstad of the Lederle Laboratories.

basic medium in flask No. 1 was added only Factor IIB″ (Cu and Fe salts). To each of the other three flasks, besides Factor IIB″, was added a combination of certain essential amino acids plus serine in half the amounts used by Kidder and Dewey (1949): to No. 2 was added "Group X," consisting of dl-serine, l(+) arginine, and dl-valine; to No. 3 was added "Group Y," comprising dl-serine, l(+)histidine HCl, l(+)lysine HCl, dl-methionine, l(−)tryptophane; to No. 4 was added "Group Z," composed of dl-serine, dl-phenylalanine, l(−)leucine, dl-isoleucine, and dl-threonine. The basis for this grouping of the amino acids was their reaction to serine (Kidder and Dewey, 1947*b*).

The life-span of Series B_7 was approximately 85 hours longer than that of Series B_6. The maximum stationary phase was also longer in three of the four cultures of Series B_7, the longest, 223 hours, occurring in flask No. 1, which contained Factor IIB″ but no amino acids. In No. 4 the average maximal population was highest, 17,000 ciliates more per milliliter than in No. 1, this being considerably higher than in Nos. 2 and 3. Logarithmic growth in No. 4 was also more rapid and more constant than in the other three cultures of Series B_7. It is of interest to note that No. 1 of this series had a 79-hour longer life-span than did No. 1 of Series B_6, also a culture of 0.25 per cent P.P., but made with Osterhout's solution and lacking Factor IIB.

DISCUSSION

Many factors besides the nutrients per se influence growth of ciliates (Calkins and Summers, 1941; Doyle, 1943). The literature treating of these factors individually, e.g., temperature and volume of media, is abundant; the conclusions are numerous and controversial. The variety of experimental techniques used and the confusion of taxonomy employed prior to Furgason's paper (1940) may be two reasons which account for these varying results. It may seem inconsistent here to attribute differences in results to dissimilar techniques and yet, further on, to compare these results with the products of a still different method; but even members of two distinct phyla exhibit certain similarities, and therefore it is legitimate to compare, at least in a broad sense, data on different species of the phylum Protozoa.

1. EFFECTS OF SINGLE NUTRIENTS

The data indicate that cessation of logarithmic growth of *T. geleii* is not the result of exhaustion of any single amino acid or growth substance used in this study. Certain of the single nutrients apparently did augment or inhibit growth to some degree, but their full effect was not discernible in 1.0 per cent proteose peptone. Findings similar to those of the present study have been made before. Isoleucine, riboflavine, and thiamine have been found stimulatory for *T. geleii* (different strains) by various workers. Isoleucine, however, has also been recorded by Kidder and Dewey (1947*b*) as completely inhibitory in the absence of serine. This seems unlikely, since in No. 2 of Series A_3 in this study, isoleucine in the absence of serine, other than that which possibly may be present in proteose peptone itself, was highly stimulatory. Here "highly stimulatory" has two meanings: statistically, it means "highly significant," i.e., the average maximal population increase of the experimental culture over that of the control could have happened by chance less than 1 per cent of the time.[4] In another sense the deviation of No. 2 of Series A_3 is highly significant because it was the only experimental culture of the single amino

[4] Cf. n. †, Table 1.

acid series which showed such a great population increase.

Previous experimental evidence differs on the effects of phenylalanine, valine, pteroylglutamic acid, and nucleic acid, which were found to inhibit growth of *T. geleii* in this study. The findings agree with those of Hall and Elliott (1935), who reported tryptophane inhibitory for *Colpidium campylum* (*T. geleii* H, Kidder and Dewey, 1945). The data on phenylalanine and valine also agree with those of Kidder and Dewey, in so far as the various forms of the amino acids can be compared. Kidder and Dewey (1947*b*) found the d-isomers of these two amino acids inhibitory for *T. geleii* W, but they make no statement about the dl-forms of phenylalanine and valine which were used in this study. Hall and Elliott (1935) have reported valine noninhibitory for *T. geleii* H. In the light of Geiger's research on "The Role of the Time Factor in Protein Synthesis" (1950), the effects of dl-threonine bear further investigation: when added at the initial growth period, it was stimulatory, but, when added at the end of the log phase, it was inhibitory. This variation, noted also in other series, indicates the desirability of investigating the addition of any of the nutrients at the end, as well as at or near the beginning, of exponential growth.

In Series A_{18}, cultures containing 0.01γ pteroylglutamic acid per milliliter showed marked decrease in maximal populations. This was surprising in view of other findings: Kidder (1946) has stated that *T. geleii* W requires a relatively high concentration of folic acid and shows a population increase in direct proportion to the amount added through the effective range. Kidder and Fuller (1946) and Jukes and Stokstad (1949) have found that 0.00065γ pteroylglutamic acid per milliliter yield half-maximal growth. The findings of Series A_{18} can hardly be explained in terms of pH, since the pH of these cultures was nearly the same as that of the riboflavine cultures in Series A_{16} in which population growth was augmented. Interpretation of apparent inhibition by nucleic acid is inadvisable at this time, since the quality of commercial preparations of yeast nucleic acid has for some time been questioned. One observation should be made about the effects of any of the single amino acids or growth substances on *Tetrahymena* populations: it is becoming apparent that *T. geleii* is highly adaptive (Elliott, 1949; Seaman, 1949). Therefore, since the stimulatory or inhibitory effects of the single nutrients were evident in the first transplant of the ciliate from unsupplemented proteose peptone, they are more important than they might appear to be.

Isoleucine and thiamine seem to enable *T. geleii* to maintain its maximal population for a longer than usual period of time (cf. Tables 1 and 2). Relatively nothing is known about the function of the amino acid isoleucine; therefore, no attempt is made to explain its action in this instance. This is not the first record that thiamine lengthens the life-span of *Tetrahymena*. Hall (1944) reported that thiamine acts as a stabilizer for *T. geleii* H, maintaining a fairly high population density for a relatively long period of time. Perhaps prolonged maximal growth and longevity in general for this ciliate are somehow bound up with fat metabolism. McKee *et al.* (1947) reported having obtained from lysed cultures of *T. geleii* W an approximately 50-50 mixture of saturated and unsaturated fatty acids, which had an average chain length of 22–24 carbon atoms. It may be that, in the living ciliate, metabolism of the fatty acids is regulated by thiamine, for that function has long been assigned to this

vitamin. Like thiamine in being a member of the B complex, pyridoxine, when added singly to 1.0 per cent P.P., produced no striking alteration of growth; still the findings of Kidder and Dewey (1949) and of this study indicate the importance of pyridoxine for *T. geleii* as Factor IIB′. This vitamin, too, may regulate *Tetrahymena* fat metabolism, since in higher animals pyridoxine is believed to aid in the conversion of excess proteins into fats and in the utilization of unsaturated fatty acids.

2. EFFECTS OF COMBINATIONS OF NURTIENTS

The foregoing conclusion that cessation of logarithmic growth is not the result of exhaustion of any single nutrient used in this study is not altogether new; according to Kidder and Dewey (1947*a*), the metabolism of *T. geleii* is more vertebrate-like than any other microörganism so far studied. It has been found that, for the proper metabolism of the vertebrates, certain nutrients must not only be present but be present in specific amounts and in appropriate combinations. Especially important are the amounts and combinations of amino acids and vitamins. Cannon (1947) emphasizes, and Geiger (1950) reaffirms, the importance of combining the amino acids. Both offer a plausible explanation for the observation that the addition of a mixture of the Kidder-Dewey ten essential amino acids and serine produced the most striking increase of population growth of *Tetrahymena* (cf. Figs. 4, 5, and 6). After working with rats which were fed two diets, each containing only five of the ten essential amino acids, Cannon reported the following: when the rats were fed the diets alternately, they quickly stopped eating and lost weight rapidly; when they were fed the diets simultaneously, their growth was noticeable. He explains: "These experiments indicate that for the purpose of tissue synthesis, all the essential amino acids must be available at approximately the same time. The concept that the utilization of the amino acids for building up tissue is a very rapid process is substantiated" (Cannon, 1947).

Although the mixture of the eleven amino acids produced the most striking increase in *Tetrahymena* growth, it was not the only combination of nutrients to augment growth. Group Z of the amino acids (cf. Table 4, Ser. B_7) was also stimulatory. This may be a matter of amount of available nutrient, since Group Z contained more amino acids by weight than did either Groups X or Y. However, if it were strictly true that the population peak was determined by the amount of amino acids, regardless of the quality, then Groups X and Y should have had populations at least higher than the control. This did not happen. Both Groups X and Y cultures had lower populations than the control. The population decrease of Group X culture is especially significant; it indicates that population growth is not determined merely by the amount of amino acids and also that this particular combination of amino acids is somewhat inhibitory.

The role played by Factor II, in whole or in part, in *Tetrahymena* metabolism is not defined by this study; at the same time, the data re-emphasize its importance for this ciliate, a fact made known some time ago by Dewey (1944).

Over and above the specific, though not conclusive, effects of the Kidder-Dewey nutrients on *T. geleii* growth, the data, as well as the observations on stock cultures, have revealed an interesting phenomenon. The findings—notwithstanding the slight variation in pH—suggest that the length of life of *T. geleii* populations varies inversely as the concentration of food: *higher* concentration

of P.P. yields *larger* individuals and *shortens* the population life-span; *lower* concentration of P.P. yields *smaller* individuals and *lengthens* the population life-span.

At present, it is impossible to give any definite figure for the cell size of the ciliates, actual measurement having been impossible in this study. It was consistently apparent during the counting process that *Tetrahymena* grown in 0.1 per cent P.P. were smaller throughout a given series than those grown in 1.0 per cent P.P. In 0.25 per cent P.P., especially in the late stages of the culture, the ciliates appeared to be larger than those in 0.1 per cent P.P. Maximal populations also showed a corresponding gradation: 1.0 per cent P.P. population > 0.25 per cent P.P. > 0.1 per cent P.P.

The growth curves give evidence that, in the three types of peptone media, the life-span itself varies inversely as the concentration of food within these limits. The cultures of 0.1 per cent P.P. had an average life-span of approximately 617 hours, as compared with the 183-hour life-span of the 0.1 per cent cultures and the 297-hour average of 0.25 per cent P.P. cultures. If pH were the cause of this variation, the life-span of the control in Series A_{20} (pH 6.88, 1.0 per cent P.P.) should have approximated that of Series A_{21} (pH 6.89, 0.1 per cent P.P.). On the contrary, the life-span of No. 1 in A_{21} was 386 hours longer (cf. Table 2) than that of No. 1 in A_{20}.

An analysis of two contrasting series in terms of total amount of cell material present for a given number of hours will better illustrate the correlation of food with cell size, population numbers, and life-span of the total population (see Table 5).

There is a possible explanation for the simultaneous occurrence in lower concentrations of proteose peptone of smaller cells, lower population, and longer life-span: overcrowding may occur in higher concentrations of P.P. and may limit the reproduction of *Tetrahymena*, just as it may limit the reproduction of other organisms (Allee, 1934), for any or all of three reasons: (1) it may increase the number of thigmotropisms (Browning, 1947) to the proximate organisms themselves or to colloidal particles of greater size and/or number; (2) it may decrease the oxygen content in a culture (Pace and Lyman, 1947); (3) it may retard the dissemination of CO_2 to the air. At all events, whatever the explanation, the findings lead consistently to the following conclusions: (1) the longevity of *T. geleii* W populations varies inversely as the concentration of food; and (2) in terms of

TABLE 5

Series	Control		Estimated Relative Cell Size	Approx. Area under Curve* (Ciliate Hours)	*T. geleii* Cell Substance (Unit Hours)
	Medium	pH			
A_{18}	1.0% P.P. (in Osterhout's solution)	6.78	1.0 *T. geleii* units†	297	297
A_{21}	0.1% P.P. (in Osterhout's solution)	6.88	0.50 *T. geleii* units†	1,254	627

* From initial growth to beginning of decline.

† Author's arbitrary expression for size of *T. geleii*.

population longevity and total cell substance, lower concentrations of P.P. provide better media for culturing this ciliate than do concentrations of 1.0 per cent and higher.

Of the total results of this investigation, it can be said, as Kidder and Dewey (1947*b*) said of one of their researches, "The results of this study raise more questions than they answer." At the same time, the data emphasize what Lwoff concluded as early as 1932: "Le problème est d'ailleurs fort compliqué à résoudre et nécessiterait le collaboration de plusieur chimistes."

SUMMARY

1. The population growth of *T. geleii* W in Proteose Peptone, Difco, to which were added, either singly or in combination, the nutrients of the Kidder-Dewey complete medium, has been studied.

2. Certain single nutrients showed some effect on population growth: dl-isoleucine and riboflavine were stimulatory; dl-phenylalanine, dl-valine, pteroylglutamic acid, and nucleic acid were inhibitory. Effects of dl-threonine bear further investigation: added at the initial growth period, it was stimulatory but, added at the end of exponential growth, it was inhibitory. This variation, noted also in other instances, indicates the desirability of studying the addition of nutrients at the end, as well as at or near the beginning, of exponential growth.

3. Isoleucine and thiamine apparently enable *T. geleii* W to maintain its maximal population for a longer than average period of time.

4. Exhaustion of specific single nutrients is apparently not the cause of cessation of logarithmic growth. Positive alteration of this phase results rather from a combination of nutrients, of which the Kidder-Dewey mixture of eleven amino acids is the most striking example.

5. The exact role of Factor II in *Tetrahymena* nutrition is not defined by this study, but its importance for this ciliate is again emphasized.

6. The significance of concentration of available food is evident; both experimental data and gross observation of stock cultures suggest (*a*) that the life-span of *T. geleii* W populations varies inversely as the concentration of food and (*b*) that, in terms of population longevity and total cell material, lower concentrations of proteose peptone provide better media for this ciliate than higher concentrations.

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CHLOROMYCETIN AND GROWTH OF CERTAIN PROTOZOA

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REPORTS dealing with the effects of antibiotics on the growth of free-living protozoans have been few. Penicillin tolerance of certain protozoans as compared with that of bacteria was reported to be relatively high by Loefer (1949). *Euglena gracilis* survived in considerably higher concentrations than did *Tetrahymena geleii*, *Chlamydomonas pseudococcum*, or *Chlorella paramecii*. Large amounts of streptomycin were also tolerated, but green flagellates when grown with this substance became colorless. Smith, Joslyn, *et al.* (1948) stated that 2.5 mg/ml of chloromycetin had no antiprotozoal effect on either *Pelomyxa carolinensis* or *T. geleii*. Since data on the response of free-living protozoans to various concentrations of chloromycetin are so meager, the following experiments were carried out.

MATERIAL AND METHODS

The organisms used in this investigation were: *T. geleii*, strains H, L-1, and L-2; *E. gracilis* var. *bacillaris*, green strain and colorless variant; and *E. viridis*. Dr. R. P. Hall kindly furnished pure cultures of *E. gracilis*, *E. viridis*, and *T. geleii*, strain H. Strains L-1 and L-2 of this ciliate were isolated locally, and the colorless variant of *E. gracilis* var. *bacil-*

laris was obtained when the green strain was cultured with streptomycin as previously reported by Loefer (1949). It has remained colorless through many subcultures, and its acid and alkali tolerances have been studied and described elsewhere by Loefer and Guido (1950). Crystalline chloramphenicol (chloromycetin) was obtained from Parke, Davis, and Company through the courtesy of Dr. E. A. Sharp. The material was dissolved to obtain appropriate concentrations in a broth medium at pH 7.1. In Series I and II the medium consisted of

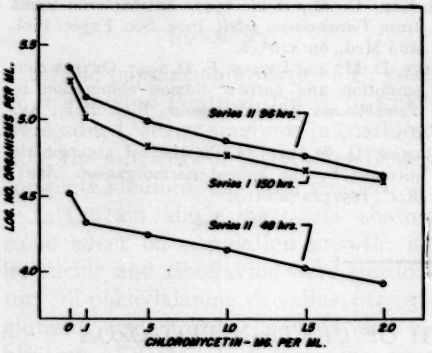

FIG. 1.—Growth of *Euglena gracilis*

1.5 per cent Bacto-Casitone and 0.01 per cent Na_2HPO_4; in the other experiments it consisted of 2 per cent Bacto-Casitone plus 0.25 per cent Bacto Yeast Extract. The media were heated to aid in dissolving chloromycetin and were filtered while hot. After Seitz filtration, respective lots of media containing different concentrations of the antibiotic were tubed aseptically in 18 × 150-mm. Pyrex culture tubes. In Series VIII and IX the media were sterilized by autoclaving. Tubes containing media were later inoculated either directly from a young, rapidly growing culture or from a dilution flask made from such a culture, in which the density of the protozoans was such that, on transfer of 0.25 ml. to each culture tube, the initial count of organisms approximated 800–1,500 protozoans per milliliter. Triplicate cultures containing 5 ml. each were set up. This is a slight modification of the procedure outlined by Loefer (1935). Cultures of Series I, II, V, and IX were incubated in a constant-temperature water bath at 27° C., the green strain under constant light and the colorless variant in subdued light. Series III, IV, VI, VII, and VIII were kept in an air-conditioned room, the mean temperature of which was 25°.5 (range 23°–29° C.). The counting procedure used has previously been described by Hall, Johnson, and Loefer (1935). Viability tests on nonmotile cultures were made by transferring a small amount of culture fluid to not less than one hundred times as much medium without the antibiotic. If viable organisms were present, free-swimming protozoans were evident within 72 hours.

EXPERIMENTAL RESULTS

Series I.—The green strain of *E. gracilis* was used. After inoculation, pH was 6.8. Tubes of media containing chloromycetin in concentrations ranging up to 2.0 mg/ml were inoculated from a 3-day stock. The initial count per milliliter was 1,260. Counts were made at 150 hours, and the cultures were observed again after 28 days. The results are summarized in Table 1 and shown graphically in Figure 1. Good growth was evident in all cultures, although progressive inhibition was evident as the concentration of chloromycetin was increased.

Series II.—The organism and the chloromycetin concentrations used were the same as in Series I. Observations are shown comparatively in Table 1 and Figure 1. In this series, as well as in Series I, chloromycetin inhibited growth. Whereas controls had increased 4,600

per cent at 96 hours, the flagellates in cultures containing 2.0 mg/ml had increased only 1,200 per cent. After 32 days, viability tests were negative for all cultures containing 1.0 mg/ml or more of chloromycetin.

shows graphically the results obtained after 48 hours of growth. Additional results are shown in Table 3. Growth of the ciliates was sharply inhibited by even the lowest concentrations of the antibiotic used. After 48 hours, control organisms

TABLE 1

SERIES I AND II: EFFECT OF CHLOROMYCETIN ON *Euglena gracilis*

CHLOROMYCETIN (MG/ML)	SERIES I		SERIES II			
	Thousands per Ml. after 150 Hours	Observations at 28 Days*	Thousands per Ml.		Observations*	
			48 Hr.	96 Hr.	23 Days	32 Days
0.0	184.0	m	34.2	204.0	m	m
0.1	113.9	m	22.3	145.0	m	m
0.5	70.1	m	18.0	102.0	m	m
1.0	62.3	v	14.2	74.9	v	n
1.5	49.5	v	11.5	61.2	v	n
2.0	40.3	n	8.9	44.3	v	n

* m = motile; v = viable; n = not viable.

Series III.—The colorless strain of *E. gracilis* was used for this experiment. A series of cultures containing chloromycetin was set up in which the initial count was 722 flagellates per milliliter. Counts were made at 6 days. The results are shown in Table 2 and Figure 2.

Series IV.—The media used were aliquots of those used for Series III. Inoculations were made at the same time as those of Series III, but from a 6-day stock of *E. viridis*. The initial count was 760 per milliliter. Results obtained after 7 days and 11 days of growth are recorded in Table 2 and Figure 2. The higher concentrations of chloromycetin inhibit growth of *E. viridis* relatively more than they retard that of *E. gracilis*, the faster-growing species.

Series V.—*Tetrahymena geleii*, strain L-2, was used to inoculate aliquots of the media used in Series II. The stock culture was 48 hours old, and the initial count was 1,875 per milliliter. Figure 3

TABLE 2

EFFECT OF CHLOROMYCETIN ON GROWTH OF *Euglena:* SERIES III, *E. gracilis*, COLORLESS; SERIES IV, *E. viridis*

CHLOROMYCETIN (MG/ML)	THOUSANDS OF FLAGELLATES PER MILLILITER		
	Series III	Series IV	
	6 days	7 days	11 days
0.0	149.6	32.4	138.0
0.03	138.5	29.6	139.1
0.06	134.8	28.9	140.0
0.30	128.2	30.1	108.8
0.60	134.0	26.5	95.0
0.90	133.0	17.9	72.0
1.20	131.5	11.3	57.5
1.50	131.0	10.0	44.2
1.80	120.4	8.4	33.7
2.10	106.6	7.0	28.5
2.28	96.2	6.4	24.6
2.45	94.0	6.3	13.5
2.62	94.0	5.8	6.5
2.80	90.6	5.3	5.7
2.98	79.4	3.6	4.4
3.15	79.8	1.9	5.4
3.32	73.8	1.4	1.4

had increased 3,800 per cent, as compared with an increase of less than 300 per cent in the presence of 100 μg/ml of chloromycetin. After 96 hours there were fewer organisms in this medium than had existed at 48 hours. Viability tests after 3 days were negative for all concentrations of chloromycetin above 0.1 mg/ml.

Series VI.—Tetrahymena geleii, strain H, was used to inoculate media, aliquots of which had been used for Series III and IV. The inoculum was obtained from a 48-hour culture, and the initial count per milliliter was 920. Ciliate counts were made after 30 and 72 hours of growth. The results are given in Table 4, and the

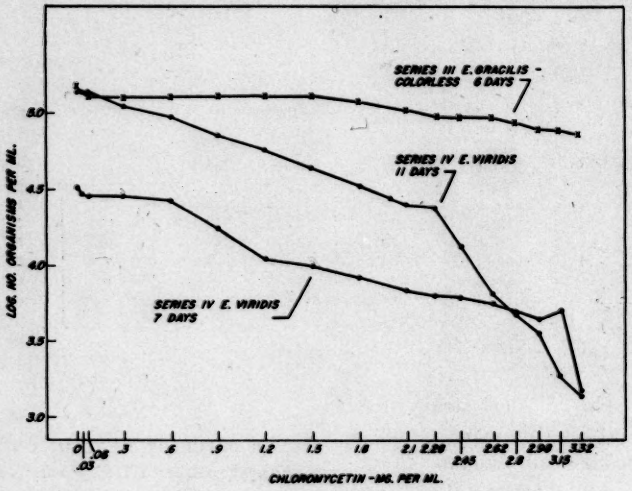

FIG. 2.—Effect of chloromycetin on *Euglena*

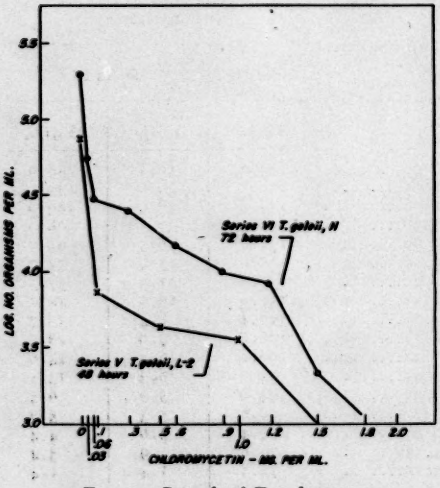

FIG. 3.—Growth of *Tetrahymena*

TABLE 3

SERIES V: EFFECT OF CHLOROMYCETIN ON *T. geleii*, L-2

CHLOROMYCETIN (MG/ML)	THOUSANDS OF CILIATES PER MILLILITER		OBSERVATIONS*	
	At 48 Hr.*	At 96 Hr.	23 Days	32 Days
0.0.....	74.0	87.0	m	n
0.1.....	7.3	5.8	n	n
0.5.....	4.3	n	n	n
1.0.....	3.6	n	n	n
1.5.....	n	n	n	n
2.0.....	n	n	n	n

*m = motile; n = not viable.

72-hour count is plotted graphically in Figure 3. Chloromycetin concentrations of 30 and 60 μg/ml sharply retarded

growth. In all cultures containing chloromycetin, counts were somewhat lower after 72 hours than they were at the 30-hour period. A similar phenomenon was noted in Series V with strain L-2 of this species. Viability tests made at 7 days indicated that 60 μg/ml are sufficient to destroy the ciliates, but they remained alive much longer in the medium containing 30 μg/ml of chloromycetin.

Series VII.—Tetrahymena geleii, strain H, was used. Several dozen control cultures were established in a medium without chloromycetin, and an equal number in broth containing 100 μg/ml. The inoculum was obtained from a 24-hour stock, and the initial count was 950 per milliliter. Ciliate counts, beginning at 12 hours and continuing at regular intervals, usually 6 hours, are graphically summarized in Figure 4. A growth peak in cultures containing 100 μg/ml of chloromycetin was reached somewhat earlier than in control cultures. In general, cultures containing chloromycetin supported less growth than did media without this substance. The declining phase began sooner and the length of the growth cycle was shorter than in cultures without chloromycetin.

Series VIII.—The purpose of this series was to compare the chloromycetin tolerance of the green strain and of the colorless variant of *E. gracilis*. Duplicate sets of cultures were set up, with chloro-

TABLE 4

SERIES VI: EFFECT OF CHLOROMYCETIN ON *T. geleii*, STRAIN H

CHLOROMYCETIN (MG/ML)	THOUSANDS OF CILIATES PER MILLILITER		OBSERVATIONS AT 7 DAYS*
	At 30 Hr.*	At 72 Hr.	
0.0	210.0	280.0	m
0.03	57.0	38.6	m
0.06	30.5	21.1	n
0.30	24.8	14.5	n
0.60	14.9	11.3	n
0.90	10.2	7.60	n
1.20	8.60	5.45	n
1.50	2.25	1.28	n
1.80	n	n	n

* m = motile; n = not viable.

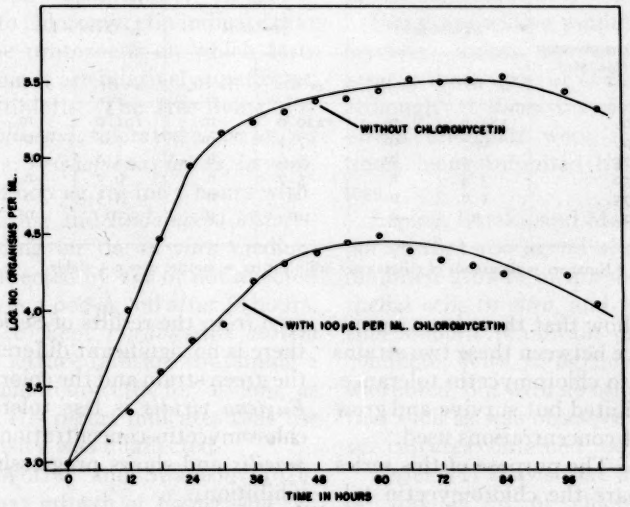

FIG. 4.—Series VII. Growth of *Tetrahymena geleii*, H

mycetin concentrations ranging from 2.0 to 3.32 mg/ml. The initial count for cultures inoculated with the colorless variant was 820, and 1,170 per milliliter for the green strain. Growth determinations, made at 6 days, are recorded in Table 5.

TABLE 5

SERIES VIII: EFFECT OF CHLOROMYCETIN ON *E. gracilis* VAR. *bacillaris*

CHLOROMYCETIN (MG/ML)	THOUSANDS OF FLAGELLATES PER MILLILITER AT 6 DAYS	
	Colorless Strain	Green Strain
0.0	54.0	72.5
2.00	46.0	63.0
2.25	41.6	54.8
2.50	43.0	56.5
2.75	42.3	56.3
3.00	37.5	49.7
3.25	31.2	49.3
3.32	29.5	46.2

Media containing chloromycetin concentrations, ranging from 1 through 2 mg/ml, were inoculated with strains H, L-1, and L-2. The initial count per milliliter for L-2 was 1,110; 940 for H; and 900 for L-1. After 40 hours of incubation, growth was recorded as in Table 6. No organisms were viable in media with 2.0 mg/ml chloromycetin. After 10 days no cultures with more than 1.0 mg/ml of chloromycetin were viable.

DISCUSSION

In these experiments the concentrations of chloromycetin to which protozoans were exposed ranged from 30 to 3,320 μg/ml. Both the green strain and the colorless variant of *E. gracilis* grew well over this entire range. Growth of the colorless form of *E. gracilis* is only slightly inhibited with concentrations up to 2,000 μg/ml, but growth is progressively inhibited above this point. It is seen from the results of Series VIII that there is no significant difference between the green strain and the colorless variant. *Euglena viridis* is less tolerant of high chloromycetin concentrations than is *E. gracilis* and shows progressively greater inhibition.

TABLE 6

SERIES IX: EFFECT OF CHLOROMYCETIN ON SEVERAL STRAINS OF *Tetrahymena geleii*

CHLOROMYCETIN (MG/ML)	STRAIN L-2*		STRAIN H		STRAIN L-1	
	40 Hr.	72 Hr.	40 Hr.	72 Hr.	40 Hr.	72 Hr.
0.0	185.0	m	226.0	m	191.0	m
1.0	9.7	m	7.4	m	7.8	m
1.25	6.2	m	5.6	n	5.8	n
1.50	3.4	n	3.7	n	6.2	n
1.75	1.6	n	n	n	3.5	n
2.0	n	n	n	n	n	n

* Numbers = thousands of ciliates per milliliter; m = motile; n = not viable.

The results show that there is no significant difference between these two strains with respect to chloromycetin tolerance. Both are inhibited but survive and grow in the highest concentrations used.

Series IX.—The purpose of this series was to compare the chloromycetin tolerance of several strains of *T. geleii*. *Tetrahymena geleii*, on the other hand,

is much more sensitive to the antibiotic and failed to survive 70 hours in media with more than 1.5 mg/ml of chloromycetin. Concentrations as low as 30 μg. sharply inhibited growth. Viability tests made after 7 days indicated that the species did not survive in a medium containing 60 μg/ml of the antibiotic, although dense cultures developed in the 30-μg. concentration. The results obtained in Series IX indicate that there is little, if any, difference between strains H, L-1, and L-2 with respect to chloromycetin tolerance.

Smith, Joslyn, *et al.* (1948) reported that 2.5 mg/ml of chloromycetin in 2 per cent Proteose Peptone, Difco, at pH 6.8 exhibited no antiprotozoal activity for *T. geleii* and "the appearance of observable activity . . . remained unchanged over a 48-hour period" (p. 432). Our results are quite different, since none of the strains of *T. geleii* tested survived as long as 48 hours in media containing as much as 1.8 mg/ml of chloromycetin.

Reports by the above-mentioned investigators and by McLean, Schwab, *et al.* (1949) on susceptibility of microorganisms to chloromycetin indicate that most of the protozoans on which tests have been made are relatively unaffected by the antibiotic. The free-living *Pelomyxa carolinensis* tolerated 2,500 μg/ml for 48 hours; *Trichomonas foetus, in vitro*, withstood 2,000 μg/ml for 7 hours without any deaths; and *Endamoeba histolytica*, depending on the *in vitro* medium used, was affected by 250 or not affected by as much as 1,000 μg/ml after 48 hours. Brown (1950) kept *Paramecium aurelia* in a baked lettuce infusion containing 2 mg/ml of chloromycetin for as long as 9–14 days. His report indicates that the culture density was unaffected.

Smith, Worrel, and Swanson (1949) reported that growth of *Escherichia coli* was stimulated by concentrations of 0.1–0.2 μg/ml chloramphenicol, but concentrations higher than 0.4 were inhibitory. McLean, Schwab, *et al.* (1949) reported that most bacteria are inhibited by concentrations below 75 and many by as little as 0.5 μg/ml. Some *Clostridium* species, however, tolerate more than 500 μg/ml. There is, however, considerable difference in strains of the same species. These investigators point out the case of *C. tetani*, one strain of which was sensitive to less than 0.1 μg/ml. Gocke and Finland (1950) have recently reported on the development of chloramphenicol-resistant variants of *Klebsiella pneumoniae*, in which resistance increased from 3.1 to 800 μg/ml. They also isolated a chloramphenicol-dependent variant. This is an unusual adaptation, although bacterial variants requiring streptomycin have frequently been obtained (Kushnick, Randles, *et al.*, 1947; Iverson and Waksman, 1948; Paine and Finland, 1948; Yegian and Budd, 1948). Streptomycin-utilizing strains of fungi have also been reported (Campbell and Saslaw, 1949; Loefer, Bieberdorf, and Weichlein, MS).

Fungi are seldom inhibited by chloromycetin, unless the concentration is greater than 1,000 or even 2,500 μg/ml, although *Actinomyces bovis* and *Nocardia asteroides* were striking exceptions, being inhibited by 20 μg/ml or less.

Lépine, Barski, and Maurin (1950) reported that 100 μg/ml of chloromycetin inhibited growth of fibroblasts and epithelial cells *in vitro*, and with ten times this amount inhibition of growth was complete. With 10 μg/ml a slight effect was noted, but with no cellular degeneration such as was observed when the tissue cultures contained 100 μg/ml.

Series VII shows that in the presence of 100 μg/ml of chloromycetin the growth cycle is shorter than in the

growth control medium; the declining phase begins earlier and death of the ciliates occurs rather rapidly. The action of chloromycetin on *Tetrahymena* appears to be cytocidal rather than cytostatic, since viability tests made a few hours after motility ceased were negative.

Smith, Worrel, and Swanson (1949) conclude from various studies that chloromycetin inhibits esterase action and in this manner effects antibiotic action on living cells. Previous studies by Smith and Worrel (1949) had indicated that this antibiotic does not inhibit proteolytic enzyme systems.

Toleration toward chloromycetin may be correlated with the production of an enzyme capable of hydrolyzing the substance. Smith, Worrel, and Lilligren (1949) actually isolated a proteolytic enzyme capable of hydrolyzing the amide linkage of chloramphenicol. The best sources of this enzyme were filtrates from cultures of *Proteus vulgaris* and *Bacillus subtilis*. Smith and Worrel (1950) reviewed the routes of chloromycetin decomposition by bacteria, isolated a number of derivatives, and found that the final products are usually amino or nitrobenzaldehydes or acids.

The protozoans, being more resistant organisms than bacteria, might be suitable tools for further investigations of the biochemistry of chloromycetin.

SUMMARY

The following protozoans were cultured in media containing various concentrations of chloromycetin: *T. geleii*, strains H, L-1, and L-2; *E. gracilis* var. *bacillaris*, normal green strain and a colorless variant; and *E. viridis*.

Good cultures of *E. gracilis* (colorless) developed in all concentrations tested up to and including 3.32 mg/ml when the basic medium consisted of casitone and yeast extract. Growth was inhibited only slightly by concentrations of 30–1,500 μg/ml. Amounts of 2,000 μg/ml and higher depressed growth moderately. There appeared to be little difference when the green strain was used. In a medium without yeast extract, the green strain seemed to be less tolerant to the antibiotic.

Amounts of 1,000 μg/ml and higher markedly depressed the growth of *E. viridis*, although only slight inhibition was evident in the range from 30 to 600 μg/ml.

All strains of *T. geleii* were sharply inhibited by 1,000 μg., and strain H by as little as 30 μg/ml of chloromycetin. The highest concentration in which living ciliates were seen after 40 hours was 1,750 μg/ml. Strain L-2, in a slightly different medium, did not tolerate as much and at 96 hours did not survive in 500 μg/ml, although it was viable in the 100 μg. cultures. After 7 days, the H strain was motile in 30, but not viable in 60 μg/ml. In general, there appears to be little difference in the chloromycetin tolerance of the three strains, but this problem needs further investigation. Maximum growth of *T. geleii* is attained in a medium containing 100 μg/ml of chloromycetin at about 70 hours.

The results are compared with chloromycetin tolerance reported for other protozoans, bacteria, fungi, and tissue cultures. It is suggested that the protozoans may be suitable for investigations on the biochemistry on chloromycetin.

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RESPIRATION OF RAT BRAIN AND LIVER AT TEMPERATURES ABOVE 37° C.

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APPARENTLY many more *in vitro* respiratory studies on tissues have been directed at temperatures below than above 37° C. Concerning the latter, Evans (1923), in a series of experiments, studied the respiration of the guinea-pig uterus and concluded that the maximum oxygen usage was at 43° C. "Heat paralysis" occurred at 49° C. Marsh (1934) reported on studies of liver and kidney respiration in the rat, rabbit, cat, and dog at several temperatures above 37° C. Small increments in respiratory rate were obtained fairly uniformly for each degree of temperature rise. Dixon (1936) reported that the respiration of rabbit cerebral cortex increased but slightly until 45° C. The rates of

respiration and aerobic glycolysis of slices of the brain cortex at 42° C. were only slightly higher than those at 37° C. In the following study the oxygen consumption of brain and liver slices from the rat has been measured over a temperature range of 5° above 37° C. Measurement was made during a 2-hour period. The cell environment was a Krebs-Ringer-phosphate solution with glucose added.

MATERIAL AND METHODS

The apparatus used in all the experiments presented herein was the Warburg constant-volume respirometer. The method was essentially that used in the direct determination of oxygen and is standard laboratory procedure. Brain and liver tissues were obtained from thirty-one nonfasting male albino rats (Sherman strain). Body-weights ranged from 61.0 to 465.4 gm., with the majority of the animals weighing between 100.0 and 175.0 gm.

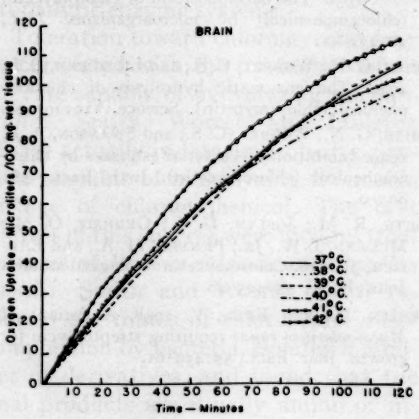

FIG. 1.—Respiration curves of rat brain at varying temperatures during 2 hours.

Each animal was killed by breaking its neck with a bone forceps. Then the liver was excised and washed in cold (about 4° C.) Krebs-Ringer-phosphate solution, which was buffered at pH 7.41. The brain was removed and washed similarly. The tissues were then placed in vessels containing Krebs-Ringer-phosphate solution on cracked ice. Slicing was accomplished with a safety-razor blade. The time from the death of the animal to the beginning of the equilibration period was about 25–30 minutes.

In each flask was placed 0.2 ml. of 10 per cent potassium hydroxide solution (central well), 2.1 ml. of Krebs-Ringer-phosphate solution, and 0.2 ml. of 0.01 M glucose solution. Tissue weights ranged from 129.8 to 257.8 mg., with the majority between 150.0 and 200.0 mg. Each tissue preparation was subjected to one experimental temperature only. Temperature regulation of the water bath was maintained with a mercury-ether thermostat ±0°.01 C. After the tissue had been sliced, the flasks containing fluid and tissues were equilibrated in the water bath for 15 minutes at the selected experimental temperature maintained during that phase of the study. The gas phase was air. Marsh (1934) reported that respiration increased slightly when serum was substituted for Ringer's solution and air was substituted for oxygen. The manometers were oscillated 105 times per minute through a distance of 6.5 cm. Readings were taken at intervals of 10 minutes during a period of 2 hours. The measurement of pH at the end of several experiments indicated very little change (7.37–7.39). Calculation of oxygen consumption was made on the basis of wet weight.

RESULTS AND DISCUSSION

The oxygen-consumption values of brain and liver obtained during the 2-hour interval were plotted and are presented in Figures 1 and 2. The data for the initial 20 minutes were selected for comparison. Until this time, respira-

tion at all temperatures was practically a straight-line function. Afterward gradual sloping occurred, as shown in the figures. The values so obtained are presented in Table 1 with statistical treatment. Comparison is made with respiratory data at 37° C.

In general, the respiration of brain was decidedly higher than that of liver. It is clear (Table 1) that there was no marked acceleration in respiration of brain above 37° C. until 42° was reached. The respiratory values at 38°, 40°, and 41° C. were slightly above that at 37°. However, at 42° the increase was 29.6 per cent. This is considered significant.

The data of liver, presented in Table 1, reveal interesting differences from that of brain. At 38° C. there was an increase in oxygen consumption of 18.9 per cent, in contrast to brain, which decreased 0.5 per cent at this temperature. Also there were increases with liver of 23.4 per cent at 41° C. and 41.1 per cent at 42°. The rises in oxygen consumption at these latter two temperatures are considered significant. It is apparent, then, that the

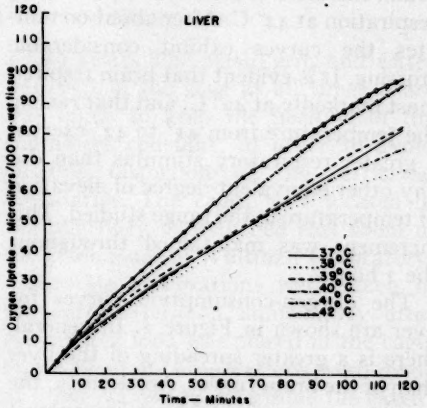

FIG. 2.—Respiration curves of rat liver at varying temperatures during 2 hours.

TABLE 1

OXYGEN CONSUMPTION FOR INITIAL 20 MINUTES

No. of Flask Determinations	Temperature (° C.)	Mean Oxygen Consumption (Microliters)*	Per Cent Plus or Minus	σ_m	P†
			Brain		
23	37	25.03	.....	±1.20	.....
19	38	27.05	+ 8.1	±1.46	>0.05
19	39	24.91	− 0.5	±1.20	>0.05
19	40	26.48	+ 5.8	±1.05	>0.05
19	41	26.35	+ 5.3	±1.05	>0.05
18	42	32.44	+29.6	±0.90	<0.01
			Liver		
24	37	16.87	.....	±0.98	.....
19	38	18.00	+ 6.7	±1.03	>0.05
19	39	20.07	+18.9	±1.22	>0.05
19	40	17.35	+ 2.8	±0.75	>0.05
20	41	20.82	+23.4	±0.61	<0.01
18	42	23.80	+41.1	±1.23	<0.02

* Microliters expressed per 100 mg. of wet tissue.

† Probability value obtained from comparison with oxygen consumption data at 37° C.

respiration of liver was accelerated earlier than that of brain with elevation of temperature.

It may be observed in Figure 1 that there is close grouping of all curves for brain, exclusive of the curve representing respiration at 42° C. After about 60 minutes the curves exhibit considerable crossing. It is evident that brain respired most markedly at 42° C. and that raising the temperature from 41° to 42° exerted a greater respiratory stimulus than did any other equivalent degree of elevation of temperature in the range studied. This increment was maintained throughout the 2 hours.

The oxygen-consumption curves for liver are shown in Figure 2. In general, there is a greater spreading of the liver than of the brain curves. Apparently, the oxygen consumption of liver, in contrast to brain, was stimulated more by the temperature rise. Nevertheless, there is, to some degree, close grouping of the liver curves in Figure 2, except those representing respiration at 41° and 42° C. At 41° there was a marked elevation of liver oxygen consumption. Again at 42° the same occurred. However, the acceleration in rate of respiration of liver during the 2 hours was greater on raising the temperature from 40° to 41° than from 41° to 42°. It is apparent from these curves that the liver respiratory rate was accelerated earlier than that of brain.

It will be noted that at the end of 2 hours at 41° C. the respiration of liver was of about the same magnitude as that of brain. Apparently, this condition existed because the liver respiratory rate either was accelerating or had accelerated at this temperature, whereas the brain had not. Also, the elevation of liver respiratory rate from 40° to 41° C. over the 2 hours was greater than that of brain from 41° to 42° over the same time interval.

SUMMARY

The respiration of rat brain and liver was studied by tissue manometric technique at temperatures ranging from 37° to and including 42° C. Glucose was added to the fluid medium surrounding the tissue. The interval of measurement was 2 hours. Elevation of temperature from 37° to and including 41° C. resulted in no marked increase in the respiratory rate of brain. However, at 42° brain showed a marked significant increase. Liver showed a marked significant increase in respiratory rate a degree earlier than brain, that is, at 41°. Thus it is concluded that liver respiration was accelerated earlier. In addition, there was some indication that liver respiration, in general, was stimulated more than brain by temperature rise.

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DOMINANCE-SUBORDINATION AND TERRITORIAL RELATIONSHIPS IN THE COMMON PIGEON

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DOMINANCE-subordination relationships in groups of animals are regarded as an important phase of behavior study. Another pattern conspicuous in certain animal societies is that of territoriality. Since the possession of territory must involve its establishment and protection, one sees immediately that some manifestation of aggression must also be present. These patterns are exhibited well together in the common captive pigeon. These birds, therefore, are particularly suitable for observing these phenomena and for attempting to determine how territoriality may influence the aggression pattern.

ANIMALS AND THEIR TREATMENT

Twelve sexually mature pigeons obtained from a dealer were used. Operations revealed seven males and five females. Colored leg bands provided means of identification. The birds were divided into three flocks of four birds each. Flock I was composed of four females, Flock II of three males and one female, and Flock III of four males. Each flock was in a cage approximately 28 inches wide, 28 inches deep, and 48 inches long. There was a roost, 5 inches wide and 3.5 feet long, located 4 inches from the back and 12 inches from the top of the cage. Scratch food, such as is used for chickens, was placed in celluloid containers, the openings of which were about 3 inches square, at the middle of the front of each cage for about ½ hour each day. Mash and grit were in similar containers about a foot away. The mash, grit, and water were left in the cage at all times. Care was taken to keep the position of the containers constant, in order to avoid possible distortion of the territorial boundaries. The cages were located in the relatively undisturbed east room of the greenhouse of Whitman Laboratory.

Routine observations were taken in the early afternoon immediately after the scratch food was placed in the cage. Supplementary observations were made at other periods to determine the extent of aggression at other times and to establish the territorial limits of each bird. Because the observer felt that her presence might affect the behavior of the birds at first, she decided to watch them through a small hole cut in a brown burlap screen. Although possibly helpful at first, this procedure seemed unnecessary during the last month of the experiment and was abandoned. The same flock arrangement was preserved throughout the entire period of study, making it possible to observe any shifts in dominance and territory which occurred. Because these fluctuations were sometimes very short-lived and apparently insignificant, it seemed wise to combine the records for the entire period and make such comments on changes in rank and territory as seemed appropriate in the presentation of the data.

PRESENTATION OF DATA

DOMINANCE-SUBORDINATION

None of these pigeons was entirely free from receiving pecks, nor were there any which were never seen to give any.

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The fighting might be severe or mild. Much aggressive behavior consisted only of coos and threats. On the other hand, pecking encounters could be so severe that the life of the weaker bird was endangered. Several other manifestations of fighting behavior were seen. Wing-flapping was a common type of aggression, which some birds used more than others. In the more vigorous fights one bird might drag another by the back of the neck over the entire cage, or often they grasped each other by the beak and struggled together. An aggressive individual would sometimes jump or lunge upon its victim. Minor idiosyncrasies, such as repeated attacks on the foot or tail of another pigeon, occurred occasionally. Defeated pigeons were seen beating their wings and squeaking as squabs do. A number of clear avoidance reactions were shown by birds when a higher-ranking individual approached. No count of these reactions was made because they could not always be determined accurately.

TABLE 1

TOTAL AGGRESSION OF THE BIRDS IN EACH FLOCK

Flock	Bird	Total Encounters	Per Cent of Aggressive Encounters	Rank
I.....	B	1,023	72.9	1
	RW	829	52.4	2
	BW	222	44.1	3
	GG	746	17.6	4
II.....	RR	2,593	55.8	1
	Wb	2,554	55.6	1
	RG	743	41.5	3
	WW	389	5.4	4
III.....	RY	1,262	84.7	1
	YW	1,179	77.9	2
	GW	1,503	23.2	3
	BY	1,868	7.2	4

AGGRESSIVE BEHAVIOR OF THE INDIVIDUAL BIRDS

Because the pattern of peck-dominance in pigeons has received full and careful attention (Masure and Allee, 1934), details of this behavior may be omitted here. It is necessary, however, to present some information concerning the rank of the particular birds involved. In Table 1 all the observed encounters of the birds are summarized. Because most fighting consisted of a rapid exchange of pecks rather than prolonged struggles, each peck has been considered an encounter, and the pigeon that gave the peck has been considered the aggressor. The pigeons were given their respective ranks on the basis of the percentage of encounters in which they gave the peck. There was a great difference in the number of encounters and in the relative aggressiveness of the individual. In two of the three flocks observed, more encounters were recorded for the more aggressive birds than for the others.

AMOUNT OF TIME SPENT IN AGGRESSIVE BEHAVIOR

Since the dominance-subordination pattern appears to be so significant in a flock of pigeons, it is of interest to note how much time is actually spent in aggressive behavior. In order to obtain any accurate data on this point, it is necessary to establish certain criteria for what is being included in the term "aggression." In addition to actual fighting and pecking, periods in which birds rapidly exchanged threats and also periods of aggressive cooing were considered here. Although the cooing done before and after fighting is sometimes difficult to tell from the cooing done in courting, acquaintance with the birds made it possible to distinguish the aggressive cooing with a high degree of certainty. Each period of

observation was ½ hour long. The timing of aggressive behavior was always done with a cumulative interval-timer. "Student's" method of paired comparsons was used in the analysis of statistical significance.

The amount of time spent in aggressive behavior under different conditions is summarized in Table 2. The first two sets of comparisons show the difference between the amount of fighting in the cage of males (Flock III) and that in the cage of females (Flock I), both with and without food. Whether food was present or not, significantly more fighting was done by the males than by the females.

The first of these comparisons, which cover samples from all the daylight hours in which the pigeons had no food, show a *P*-value of 0.025; this is considerably less than 0.05, which is the largest number to be considered statistically significant. Immediately after food was put into the cage, the fighting became much greater; the difference between the sexes has still greater statistical significance, with a *P*-value of 0.0002. The last set of comparisons merely shows that the increased amount of fighting during the feeding time was highly significant statistically.

TABLE 2

AMOUNT OF TIME SPENT IN AGGRESSIVE BEHAVIOR

Type of Observation	Per Cent Time Spent Fighting	No. of Half-Hour Observations	Mean No. of Seconds Fought per Half-Hour	Difference in Means	*P*-Value
Males (Flock III), without food	4.75	17	85.59	78.53	0.0024
Females (Flock I), without food	0.39	17	7.06		
Males (Flock III), with food (early P.M.)	17.19	14	309.57	267.57	0.0002
Females (Flock I), with food (early P.M.)	2.33	14	42.00		
All birds with food (early P.M.)	11.13	22	200.41	134.41	0.0012
All birds without food (early P.M.)	3.67	22	66.00		

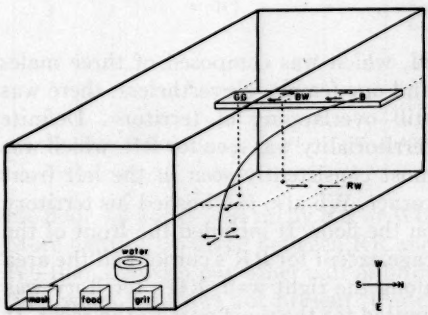

FIG. 1

TERRITORIALITY

The maps presented here are based on half-hour observation periods, which represent every hour during the day and were distributed throughout most of the months of the experiment. Each time a pigeon was seen in a certain area, a notation was made. By combining these observations it was possible to determine the territorial range of each bird.

The graph for Flock I (Fig. 1), which was entirely composed of females, indicates by dotted lines the boundaries of the territories in which each bird was normally seen. Some birds showed a rather marked tendency to move in a certain direction from their established territories, and these movements have

been indicated by solid arrows. In one case, after two birds (GG and BW) formed a homosexual pair, they moved to a territory which was new to both of them. This movement was shown with dotted arrows to indicate that it was different from the ordinary movements out of a territory. RW was most often seen in the back right-hand corner of the floor. B had less definite territory boundaries than any other bird.

The territorial relationships were more clear cut, as shown in Figure 2, in Flock

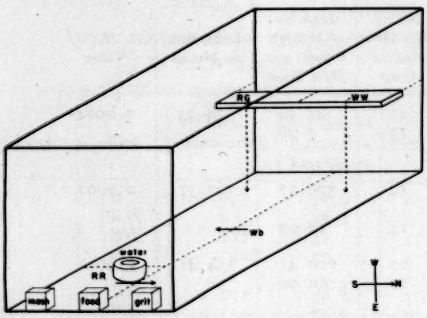

FIG. 2

II, which was composed of three males and one female. Nevertheless, there was still overlapping of territory. Definite territoriality was seen for RR, which was most consistently seen in the left front corner. Wb also established his territory on the floor. It included the front of the cage except for RR's corner and the area along the right wall. RG's territory was limited for the most part to the roost. It is interesting to note that in the few cases when he was seen on the floor he avoided the territory of RR and Wb. At first, WW stayed almost entirely on the roost, but toward the end of the observations she began to be permitted on the floor because the males courted her there.

The territorial relationships in Flock III (Fig. 3), which was composed entirely of males, were the most diagrammatic of all. GW was always seen on the left part of the roost, and BY, except for a brief period in which he almost established himself on the floor, occupied the rest of the roost. RY and YW together possessed the entire floor. They were usually seen close to each other. Apparently YW was sexually attracted to RY.

RELATIONSHIPS BETWEEN DOMINANCE-SUBORDINATION AND TERRITORIALITY

Inasmuch as the definition of "territory" has been accepted as the defense of a certain area, it becomes necessary to correlate the data on aggressiveness and territoriality. Table 3 represents pair-contacts which were observed in different areas in the cage of females at feeding time. Similar records were kept for all three flocks both at feeding times and under normal conditions and are the basis for the following remarks about

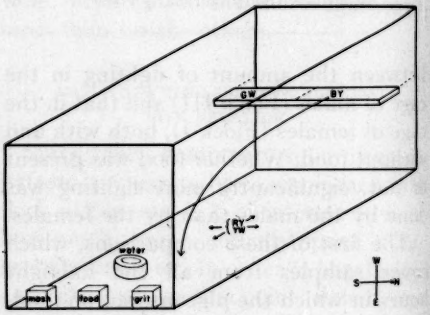

FIG. 3

pair-contacts in different parts of the cages.

During feeding time the area on the floor near the food was the center of conflict in Flock I. Table 3 shows that RW, the one bird established exclusively on the floor, gave considerably more pecks and threats (339) at feeding time than the other birds combined (126), but her

aggressiveness never extended beyond the floor.

The roost was the major area of conflict in Flock I when no food was present. The number of encounters on the roost (76) was greater than for the rest of the cage combined (52). BW and B, which controlled territory on the roost, showed more aggression there than the other birds did. RW was definitely limited to the floor, where she was relatively aggressive. RW was severely attacked or threatened on the floor by only one bird, BW. The area of most of these encounters was in or near RW's territory. Repeated observation of these encounters showed that BW frequently attacked RW if RW moved out of her corner and pursued her until she returned there. RW showed marked aggressiveness in her corner, which decreased rapidly as she got farther away from it. She was the only bird in the flock which was never aggressive on the roost.

In Flock II, also, the majority of encounters during feeding occurred on the floor around the food. The fighting was particularly accentuated between RR and Wb, which had adjoining territories near the front of the cage. About half (217) of the total number of pecks and threats (437) exchanged at this time occurred between these two birds.

When no food was present in Flock II, RG, which was firmly established on the roost, gave all but one of the 18 pecks that were given there and received none. When he occasionally ventured down on the floor, he was driven up by RR or Wb. More fighting occurred between RR and Wb than between any other pair of birds even when food was not present. Under these conditions, 112 of the 146 encounters were between these birds. WW, the female in the cage, was seen to give pecks on only one occasion, and this occurred after the routine observations had been completed.

When no food was present in Flock III, both RY and YW, whose territory was being invaded, were most aggressive, together giving 547 of the total pecks

TABLE 3

PAIR-CONTACTS IN FLOCK I AT FEEDING TIME

Birds	Floor—Front			Floor—Middle			Floor—Back			Roost			Total
	Left	Mid.	Right	Left	Mid.	Right	Left	Mid.	Right	Left	Mid.	Right	
B:GG	.....	12	.....	4	1	.....	.....	.....	.....	1	4	.....	22
:BW	.....	7	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	7
:RW	.....	9	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	9
GG:B	1	3	.....	2	.....	.....	1	.....	.....	.....	.....	.....	7
:BW	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1	.....	1
:RW	1	10	.....	8	.....	1	3	.....	.....	.....	.....	.....	23
RW:GG	.....	58	.....	17	4	4	1	5	3	.....	.....	.....	92
:B	6	97	2	29	41	2	3	3	.....	.....	.....	.....	183
:BW	25	37	.....	.....	1	.....	.....	.....	.....	.....	.....	.....	63
BW:GG	.....	1	.....	.....	1	.....	.....	.....	.....	.....	4	.....	6
:B	.....	6	.....	.....	.....	.....	.....	.....	.....	.....	12	.....	18
:RW	.....	34	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	34
Total	33	274	2	60	48	7	8	8	3	1	21	0	465

and threats (633). Neither of these birds attained any degree of dominance on the roost, although in three instances it was the site of aggression by YW against RY and GW. During the period when BY sought to establish himself on the floor, he had more encounters with YW than with any other bird, both at feeding time and in the records covering normal conditions during all hours of the day.

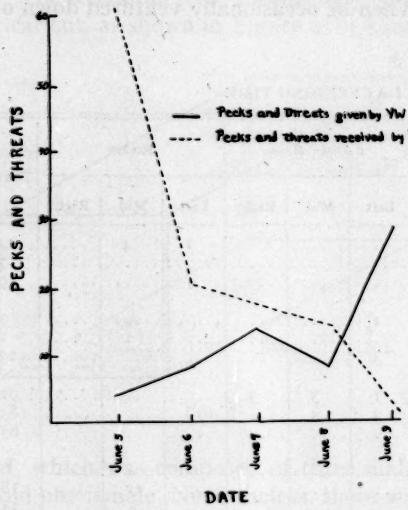

FIG. 4.—Aggressivesness of YW in Flock III

When no food was present in Flock III, YW was the aggressor in 164 cases on the floor and in none on the roost; RY was the aggressor in 23 cases on the floor, but never on the roost. The fact that YW pecked RY seemed to be associated with the sexual attraction of YW toward RY. Both RY and YW were attacked a number of times by BY at the time that the latter bird almost established territory on the middle and center of the floor. GW, a low-ranking bird, confined his aggressiveness almost entirely to his territory at the right end of the roost.

ADDITIONAL OBSERVATIONS

When the birds were first put into the final flock arrangement in Flock II, RG established himself on the roost. He was so aggressive at that time that no bird defeated him in any of his frequent encounters. His rank gradually decreased, however, until at the end of the observations he was subordinate to RR and Wb, which were established on the floor near the food.

Another interesting case is that of YW in Flock III. YW had no territory at the first of these observations. He crouched on the edge of the middle of the roost, occupying as little space as possible. GW drove him to the floor repeatedly, and by June 7 he was seen crouching in the extreme right front of the cage rather than on the roost. By June 9, YW had established himself on the floor. Paralleling these records on YW's shift in territory from June 5 to June 9, daily records, shown in Figure 4, were kept of the number of pecks he gave and received. On June 5, there was no other bird in the flock receiving as many pecks as YW; by June 12, there was none giving more. This rapid and complete reversal, however, should not be regarded as a typical change.

During the feeding period the birds in each flock that were normally present on the floor might fly up to the roost, whereas those birds normally on the roost were on the floor eating. In Flock II, particularly, such a behavior pattern was conspicuous. It was an almost daily occurrence for RR and Wb to fly to the roost after a brief period of feeding. This gave RG and WW a chance to eat. When they finished eating, they returned to the roost, and RG drove off RR and Wb. In Flock I, RW sometimes flew to the roost during the feeding period and exercised there until driven off by B. In sev-

eral cases YW in Flock III was seen on the roost during the feeding period until driven down by BY.

On several occasions food was placed on the roost instead of in its usual place on the floor. The birds established on the floor had great difficulty in staying on the roost long enough to get any food; they were almost completely subordinate to the birds they dominated on the floor. Also at the time the cages were being cleaned or when persons in front of the cage startled the birds, those established on the floor attempted in vain to remain on the roost.

DISCUSSION

Schjelderup-Ebbe (1922, 1935) described dominance-subordination relationships for many birds, both domestic and wild, presenting the peck-order in some detail. He described the typical straight-line order of dominance in chickens in which the top-ranking bird pecks all those below him without penalty. The next highest bird, in turn, pecks all those below him but receives no pecks from them in return. This same pattern, now designated as "peck-right," is maintained throughout the entire flock. Masure and Allee (1934) confirmed Schjelderup-Ebbe's conclusion about the nature of the hierarchy in hens by means of the Brown Leghorn breed. They demonstrated, in addition, that in common pigeons the typical hierarchical pattern is one of peck-dominance rather than of peck-right. In the case of peck-dominance, the more dominant birds are not free from pecks from their subordinates, but their high rank rests in the fact that they give more pecks than they receive. Bennett (1939), working on ringdoves, paralleled the observations made by Masure and Allee (1934) on pigeons. She found a similar peck-dominance situation in her birds.

The degree of dominance and hence the rank of a pigeon within a flock may vary. It was not unusual to find minor variations from day to day, and birds sometimes showed a definite change from one rank to another within several weeks. In a few cases the change was dramatic.

Diebschlag (1941) described a number of changes in rank among the pigeons he studied. The changes which he reports are, on the whole, produced by psychological devices. Most striking perhaps are the situations in which he engaged certain pigeons in combats with stuffed pigeons. By allowing a bird to defeat one of these dummies, the bird's aggressiveness could be built up enough so that it would fight its way to a higher rank in the flock. Apparently this change was effected through increased confidence and development of imposing behavior. This technique was more successful in the case of males than of females. Some of these psychological devices involved modification of territories. In one case he accustomed the birds to the presence of a large cardboard carton, and in another instance he used a hemp rope. Acquaintance with these extraneous articles placed in a cage seemed to give some birds enough of an advantage over their companions so that changes in rank followed.

The sex cycle is also suggested by Diebschlag and others as being responsible for some fluctuations in rank. There seemed to be a clear-cut example of such a change in the present observations in the case of WW, which was the only female in Flock II. For months no pecks were recorded for her, but, after the formal data were complete, WW was seen chasing other birds in the cage after she had begun to lay eggs.

It was noted in these Whitman Laboratory experiments that, in general, the

high-ranking pigeons engaged in more encounters than did their subordinates. The only flock for which this did not hold true was Flock III. In this case the picture was distorted as a result of BY's many encounters during his unsuccessful attempt to establish a territory on the floor.

Others have also reported more contacts for high-ranking animals. Masure and Allee (1934) report that high-ranking pigeons engage in more encounters than low-ranking ones do. Bennett (1941) found a similar situation in her ring-doves. Guhl (1942) recorded that in his small flocks of common domestic fowl the first in the peck-order were the first in activity rating. This correlation did not follow perfectly, however, except for the top animals. Shoemaker (1939) states in his work with canaries that birds ranking high in dominance deal more pecks than lower-ranking birds do. On the other hand, he recognizes Schjelderup-Ebbe's statement that chickens at the top of the peck-order do less pecking than those which rank lower and, by the nature of their pattern, have fewer encounters. Shoemaker suggests that the difference may be related to the relative size of living space. In some cases the low-ranking birds are better able to avoid contacts with the higher-ranking ones because of a greater living area. In the experiments reported here, the birds were so crowded that, even though some avoidance reactions occurred, it was not likely that subordinate birds could have altogether avoided contact with the dominant ones. Diebschlag made no direct statement comparing the amount of activity in different ranks but brought out the fact that, because of the constant tendency for pigeons to seek more territory, the top birds are necessarily involved in many encounters to defend their position.

The males were clearly seen to show more aggressiveness than the females. In pigeons, as in many other animals, this seems to be associated with the courtship and mating pattern. The records for all three of the Whitman Laboratory flocks showed that no female had so many total encounters or was aggressive in so high a percentage of them as the high-ranking males. Not only the total number of aggressive incidents was notably less in the females but also the amount of time spent fighting.

Although aggressive behavior in pigeons occupies only about 3 or 4 per cent of the observation time, dominance-subordination relations appear to be very important in the life of the flock. A low percentage of time is also reported for other animals. Crawford (1942), in a statistical analysis of the social activities of captive chimpanzees, found that they spent only 9.4 per cent of their time in aggressive behavior. Warne (1947), in observing small groups of caged mice, noted that the total time of all contact behavior among the mice was 1.3 per cent of the observation time and that only 0.24 per cent of the observation time was spent in aggression. The small percentage of time spent in aggression is typical of the different species studied. This may be because of the strength of an established social organization in which each animal accepts its position. Although changes in the social order occur, they are not frequent enough to cause a great amount of time to be spent in fighting.

Although numerous definitions of "territory" have been put forth, the most satisfactory is the concept accepted by Noble (1939) that any defended area may be regarded as the territory of the defending animal. There are in the literature several observations of territoriality in pigeons, especially in regard to

the nesting place. Taylor (1932) saw establishment of territory by pigeons in a pen and noted that territoriality could be affected by the addition or removal of certain birds. Masure and Allee (1934) brought out the presence of a place factor in the social organization in pigeons. Bennett (1939) mentioned territoriality in ringdoves.

Diebschlag (1941), in describing territoriality, speaks of each bird as having a small resting place that is vigorously defended against intruders. In addition, each bird has a larger sphere of influence that includes the resting place. The sphere of influence of pigeons may overlap, and the defense of these spheres is less violent than is true for resting places. If a bird is low-ranking or if the birds are very crowded, the sizes of the resting place and of the influence sphere are very likely to be identical. Probably the explanation for not seeing this distinction between the resting place and the influence sphere in the present series of observations lies in the smaller size of the cage used.

Diebschlag refers to a vertical division of territory which occurred in an experiment in which the pigeons were allowed to fly throughout an entire room. Certain birds dominated on the floor, while others dominated on the tables and above. This seems comparable to the division which occurred between the roost and the floor with the Whitman Laboratory pigeons. He noted that, in general, an individual was more aggressive in its own territory than anywhere else in the cage. The size and desirability of a bird's territory were associated with its rank.

Diebschlag recognized some changes in territoriality that appeared to occur spontaneously, but he stated that such changes were infrequent. The experimental conditions, however, were not alike, since these birds in Whitman Laboratory were probably more crowded and there were no efforts to produce artificial shifts either of territory or of social status. Both Diebschlag's work and the observations presented here indicate that territoriality as well as aggression is more deeply intrenched in males than in females.

In recapitulating the data from these present experiments in regard to the aggression which occurred in different areas of these cages, it is seen that the birds which dominate on the floor are almost completely subordinate to the others if they fly up to the roost. Those individuals which were established on the roost, in turn, were not usually successful in aggression on the floor. In these particular experiments the observations taken at feeding time gave good indications of the rank order. It might even appear that the rank of an individual bird depended largely upon the distance of its territory from the food. Certainly, the higher-ranking birds were those established on the floor, except in the cage of females (Flock I), where the dominance-subordination and territorial patterns were relatively indefinite. Even when the birds were feeding, however, some of the peck exchanges in each flock seemed to be nothing more than minor variations from the normal pattern or indications of indecision involved in an attempt to extend territory.

An approach to the peck-right situation in chickens was seen when pair-contacts within limited areas of the cages were considered. Although a pigeon did not necessarily give all the pecks in its territory and receive none, the birds' aggressiveness was much more effective if each was within its own territory. This suggests that the territorial organization in pigeons blocks a rigid dominance hierarchy, such as is found in chickens.

SUMMARY

1. The degree of peck-dominance possessed by individual pigeons in a flock is likely to fluctuate.

2. The amount of time spent fighting by a flock of male pigeons is significantly greater than that spent by a flock of female pigeons and still is very low.

3. Aggressive behavior occupies only a small amount of time, although dominance-subordination relations appear very important in the organization of territorial relations.

4. Definite territories are established by the pigeons in a flock. These territorial boundaries are not always rigid for any given time, however, and the territories themselves will shift in substance as well as in detail. Territoriality is more pronounced among male pigeons than among female pigeons.

5. There are indications suggesting a positive correlation between the percentage of encounters won by a bird and the amount of territory it possesses and is tolerated in.

6. The aggressiveness of any bird is much more effective in its own territory than in other areas of the cage.

7. Pair-contacts within the territorial boundaries show an approach to the peck-right situation in chickens.

8. The territorial organization in a flock of pigeons seems to block a rigid dominance hierarchy such as is found in chickens.

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